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Biofabrication and 3D Tissue Modeling

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Preface

Existing two-dimensional (2D) *in vitro* cell culture experiments, which are the standard for the initial screening for drug compounds, do not replicate the *in vivo* tissue microenvironment, and animal experiments, which are conducted as the gold standard for biological testing, cannot be fully relied upon to predict the response in humans. Therefore, despite the total cost involved in the drug development process exceeding \$1 billion USD, over 90% of drugs entering clinical trials ultimately fail. Thus, also driven by worldwide efforts to reduce the use of animals for biosafety testing, the research on 3D tissue modelling has been expanded into the field of biomi-croengineering. A 3D *in vitro* tissue model that can accurately capture the diversity of the *in vivo* microenvironment and the complexity of physiological or pathophysiological conditions offers the potential to better understand the possible treatment options and approaches regarding pathologies of interest. Therefore, the development of a 3D tissue model signifies a bright future for new drug development.

3D tissue modelling is an advanced modern approach in biomedical engineering. It offers the prospect of creating complex 3D tissues or organs *in vitro* by integrating technologies from engineering, biomaterials science, cell biology, physics, and medicine. Recent applications of 3D tissue modelling have provided valuable information on the current state-of-the-art in biomedical fields and in advanced biofabrication technologies.

The research into the 3D *in vitro* modelling platform has developed significantly in recent years but remains at an inchoate stage for preclinical applications. If we approach regulation in a more practical and less rigorous way in terms of both the technical and biological aspects of the development of the 3D tissue model, I believe that we can refrain from making various detours and steer this in the right direction for preclinical applications.

Therefore, the present work reviews all relevant information on the principles, fabrication technologies, applications, and future perspectives of 3D tissue modelling in a one-stop resource for academics. The book will describe the principles of 3D tissue modelling and review the fabrication methods and processes of 3D tissue models, including various microfluidics, microfabrication, and 3D bioprinting technologies. It will also describe materials such as the synthetic, natural, and tissue-derived (decellularized extracellular matrix (dECM)) bio-inks that are used for 3D tissue modelling. The review on the broad applications of 3D tissue modelling will include specific examples and case studies and will cover tissue engineering for therapeutic purposes and the development of *in vitro* tissue models to screen drugs or to study diseases. Current challenges and future perspectives of 3D tissue modelling will also be discussed. To this end, we gathered outstanding experts from each important field with the intention that this book will serve as a navigational aid for researchers who want to develop *in vitro* tissue models.

We would like to express our gratitude to all authors, the Royal Society Chemistry publishing team, and Dr Ju Young Park, Prof. Jinah Jang, and Jae Yun Kim who contributed to this book.

Dong-Woo Cho

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CHAPTER 1

Microstereolithography

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1.1 Introduction

Fabrication is a critical process in making materials into functional parts and devices. Traditional fabrication technologies such as machining and molding are commonly used in macro-scale three-dimensional (3D) fabrication. However, they are not adequate for microscale fabrication. Modern micro- and nanoscale fabrication technologies such as photolithography, soft lithography, electron beam lithography, focused ion beam lithography, dip-pen lithography, and atomic layer deposition, are often 2-dimensional (2D) in nature for thin film and surface patterning.

3D printing, as an additive free-form 3D fabrication technology, has achieved great commercial success in the past decade, because of its low cost, simplicity, and versatility. Microstereolithography is a light-assisted 3D free-form fabrication technology. This technology utilizes photosensitive materials, which can solidify upon ultraviolet (UV) or short wavelength visible light exposure. By spatially controlling the exposure dose, the desired 3D structure can be fabricated. This technology evolves in two directions. One, scanning-based microstereolithography, which provides the extremely fine resolution (sub-micron scale) but slow fabrication speed (*e.g.*, hours). Two,

[†]Contributed equally to this work.

projection-based microstereolithography, which provides both fine resolution (micron scale) and fast fabrication speed (*e.g.*, seconds to minutes).

Microstereolithography is a powerful tool for biofabrication.¹ It has successfully demonstrated its capability of fabricating a wide range of biomaterials such as hydrogels, proteins, and cell-laden materials. Both synthetic and natural materials can be used to print, each having different advantages for stability, mechanical properties, cytocompatibility, and printability. Material decisions must come from the eventual application for the printed structure, as even the choice of photoinitiator can greatly impact the print.^{2,3}

This chapter covers basic physical and chemical mechanisms in photopolymerization, materials, devices, and systems of microstereolithography. The photopolymerization mechanism is detailed in Section 2. Materials for microstereolithography are discussed in Section 3. Scanning-based microstereolithography, including single-photon polymerization microstereolithography, and two-photon polymerization nano-stereolithography, is detailed in Section 4. Projection-based microstereolithography, including liquid–air interface polymerization and liquid–substrate interface polymerization, is discussed in Section 5.

1.2 Photopolymerization

In this section, we will focus on photopolymerization, a technique most often used to crosslink liquid state monomers or oligomers into solid state long-chain polymers. Photopolymerization uses free radicals to initiate and crosslink strands within a monomer solution to form a solid hydrogel. When paired with microstereolithography techniques, various complex structures can be fabricated.^{4,5}

1.2.1 Step-growth Polymerization

Two of the main types of polymerization observed in hydrogel scaffolds are step-growth and free-radical polymerization. Although photopolymerization methods use free radicals to polymerize structures, the kinetics of step-growth polymerization can describe some of the more unique polymerizations, and thus it is important to cover.^{6,7} Step-growth polymerization occurs when polymer chains grow in a stepwise fashion either by condensation reactions, in which water is removed, or when reactive end groups interact. When considering simple linear chain reactions, the mechanisms and rates of all polymerization steps can be assumed as equal. Moreover, the Carothers equation (eqn (1.1)) defines the level of completion of the step-growth polymerization

$$x_n = 1/(1 - p) \quad (1.1)$$

where x_n is the average chain length, and p is the conversion rate of the monomers into polymers. This is an incredibly useful equation for

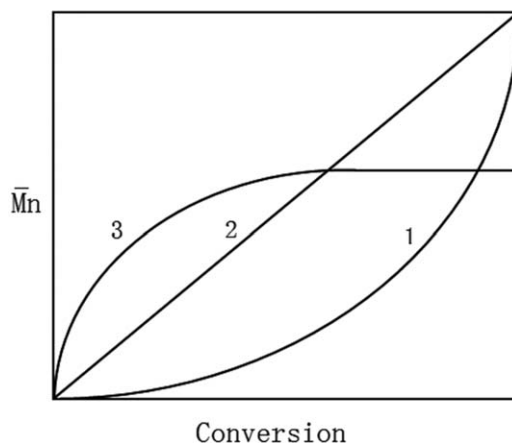


Figure 1.1 Number-average molecular weight ($\bar{M}_n$) vs. monomer conversion curves for step growth polymerization (1); living polymerization (2); and free-radical polymerization (3). Reproduced from ref. 9 with permission from The Royal Society of Chemistry.

predicting how long the reaction needs to proceed to obtain the correct molecular weight.⁸ As one can see, this defines an exponential relationship (Figure 1.1).⁹ As one might imagine, high molecular weights become increasingly more difficult to achieve for three reasons: (1) the frequency of reactive end groups meeting decreases; (2) the frequency of side reaction interference increases; and (3) when two or more monomer types are used in the reaction, it is difficult to ensure that the starting material concentrations are equal. This third point is an issue for users creating copolymer hydrogels, such as those consisting of monomer A and monomer B, where A-A does not react, nor B-B, but only A-B. To stop the reaction at a lower molecule weight, the user has a few options. One, the reaction can be rapidly cooled at the correct time point to slow down the polymerization rate as many step reactions have high activation energies. Two, a monofunctional material can be added to “cap” the end of the polymer, preventing it from further reactions. Lastly, if making a copolymer, a stoichiometric imbalance of the starting materials can be used. For example, if more A groups are used than B, eventually the polymer will have two A end groups on a chain and the B monomer will be completely consumed preventing further reactions with that polymer. The Carothers equation can be expanded to define this scenario, shown in eqn (1.2),

$$x_n = (1 + r)/(1 + r - 2p) \quad (1.2)$$

where x_n is the average chain length, p is the conversion rate of the monomers into polymers, and r is the ratio of monomer A to monomer B (N_A/N_B).⁸

1.2.2 Free-radical Polymerization

In free-radical polymerization, a free radical interacts with reactive end groups to form a polymer. The basic structure of the active group is $\text{CH}_2=\text{CR}_1\text{R}_2$, as the pi-bond in the carbon-carbon double bond allows it to be rearranged when exposed to a free radical. From here forward this will be referred to as the active center. In the polymerization scheme, there are three main steps: initiation, propagation, and termination. As will be expanded on later in the chapter, the initiation step is the activation of the active center when a free radical reacts with the carbon-carbon double bond. After initiation, the active center reacts with other double bonds, propagating the chain to form a polymer and transferring the free radical to a new active center. The termination step can then occur in several ways: (1) two active centers can react; (2) one active center and one free radical can react; (3) the active center transfers to another molecule; or (4) interaction with impurities or inhibitors. Chain transfer is another important mechanism that occurs in free-radical polymerization. This occurs when an active center collides with a molecule such as a solvent, initiator or monomer, transferring the free radical to the second species.

Free-radical polymerization can theoretically go to full conversion, but the interaction of free radicals with each other must be kept in mind. Eventually, free radicals will form covalent bonds with each other, stunting the chain propagation and preventing full conversion. As a general rule, the greater the free radical concentration, the shorter the chain length. The viscosity also has an impact on the rate of the conversion, as it impacts the diffusion of polymer chains through the medium. As the conversion increases, so does the viscosity, preventing chains from interacting and terminating the reaction at the same speed.⁸ This means the propagation of chains by free radicals increases towards the end of the reaction (Figure 1.1).⁹ This auto acceleration can be prevented by stopping the reaction before the initiation and propagation steps become diffusion-mediated. Regardless of conversion, the average chain length of the completed polymer shows little variation throughout polymerization. Moreover, longer reaction times may increase polymer yield but will not increase the chain length. Increasing the temperature can decrease the molecular mass, but the best way to control the polymer chain length is to alter the initiator concentration.⁸

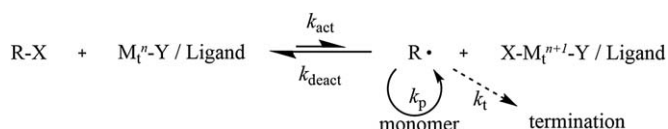
1.2.3 Living Free-radical Polymerization

In “living” polymerization, the termination step is suppressed, so that the free radical is continually recycled. This results in a much lower polydispersity. Most living polymerization methods use a reversible “cap” on the active site, preventing it from continuously reacting. This slows down the reaction and suppresses termination, which results in a steady increase in chain length, defined by the following eqn (Figure 1.1):

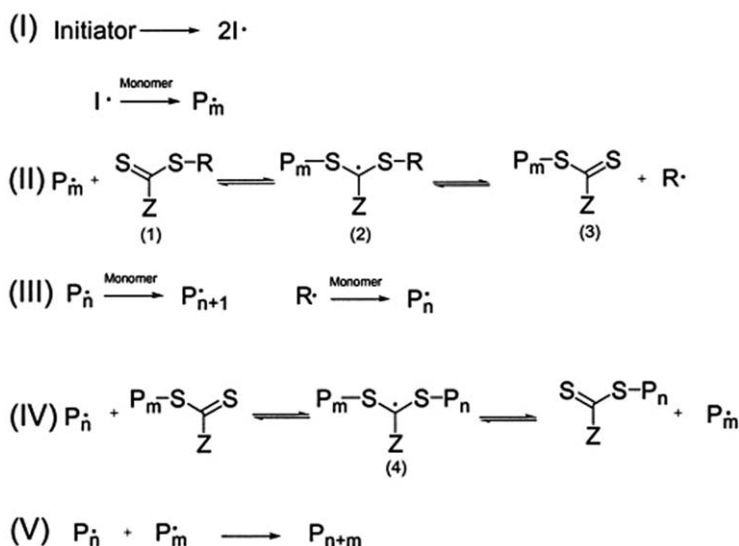
$$x_n = \frac{[\text{M}]_0}{[\text{I}]_0} \times p \quad (1.3)$$

where x_n is the degree of polymerization, $[M]_0$ is the initial monomer concentration, $[I]_0$ is the initial initiator concentration, and p is the conversion rate. Studies have been performed to try to improve reaction schemes for living polymerization.⁸ Two of the most common techniques are atom transfer radical polymerization (ATRP) and reversible addition fragmentation chain transfer (RAFT) (Schemes 1.1 and 1.2).^{10,11}

A typical ATRP reaction consists of a dormant species, such as an alkyl halide (R-X) that can be reversibly activated by a transition metal complex (Mt^{n+}/L) to form an active radical ($R^\bullet$) and an oxidized metal complex ($XMt^{n+1}Y/L$). When the free radical is not capped with the metal complex, it is free to propagate until it is capped again. ATRP methods are fairly versatile and work best with monomers such as styrenes, methacrylates, methacrylamides, and acrylonitrile. ATRP can also be used in conjunction with an initiator in a similar process called “reverse ATRP”.



Scheme 1.1 ATRP reaction scheme. A reaction consists of an alkyl halide (R-X), a transition metal complex ($Mt^{n+}Y/Ligand$), an active radical (R), and an oxidized metal complex ($XMt^{n+1}Y/Ligand$). Reproduced from ref. 10, with permission from American Chemical Society, Copyright 2001.



Scheme 1.2 RAFT reaction scheme. The dithio compound here is able to bind to up to two chains at once, rendering them dormant. Reproduced from ref. 11 with permission from John Wiley and Sons, Copyright © 2002 Wiley Periodicals, Inc.

For RAFT reaction schemes, the “capping” species contains a dithio compound and a good leaving group within its structure. The compound reacts with the free-radical functionalized polymer chain to form a dormant chain, and the leaving group is released as another free radical in solution. At an equilibrium state, the compound can react reversibly with up to two chains, rendering them dormant. This yields a much lower polydispersity. The molecular weight of the polymer can be compared to the conversion to distinguish the two types of polymerization (Figure 1.1).^{8,12} Translating to hydrogel scaffolds, where the free radicals create cross-linking between polymer backbones, this means that a lower free radical concentration is needed for living polymerization than free-radical polymerization.¹³

1.2.4 Photoinitiators

In order to create the free radicals for photopolymerization, photoinitiators are added to the prepolymer solution. The photoinitiators used in hydrogels usually generate free radicals by one of two methods: (1) photocleavage or (2) hydrogen abstraction. In photocleavage, the molecule undergoes bond cleavage at C–C, C–Cl, C–O, or C–S bonds when exposed to light, generating free radicals. In method two, the photoinitiator undergoes hydrogen abstraction from an H-donor molecule, forming a ketyl radical and donor radical. When selecting an appropriate photoinitiator, the user must consider several factors including biocompatibility, solubility in water, stability, and cytotoxicity. Photoinitiators can greatly impact the print resolution, as well as cell viability. As such, preliminary studies modulating the concentration and exposure time of the print are necessary for any new photoinitiator considered.^{14–16}

Photoinitiators can be characterized by the wavelength at which they most strongly absorb. Although less popular, visible light photoinitiators can be advantageous if the user plans on exposing the pre-hydrogel solution for a longer period of time. Compared with other photoinitiators that activate in the UV-range, encapsulated cells can be exposed for a longer time with less risk. However, these photoinitiators are more difficult to work with, as ambient conditions require the user to work in the dark. One popular visible light photoinitiator is Eosin-Y, which generates radicals by hydrogen abstraction. It is activated at 490–650 nm and is a common choice as a cyto-compatible molecule. Unfortunately, in order to generate enough free radicals, it often needs a coinitiator (*e.g.*, triethanolamine (TEOA)) and a comonomer (*e.g.*, 1-vinyl-2 pyrrolidinone (NVP)) included in the prepolymer solution, making modulation of concentrations more difficult to work with than some of the common UV-initiators.^{17,18}

UV-activated photoinitiators can be advantageous for users who desire easier ambient conditions. However, because the pre-hydrogel solution is exposed to UV, care must be taken to limit the exposure time when working with encapsulated cells in the gel. Preliminary exposure studies with cells are

recommended to ensure limited cell death. Two common cytocompatible photoinitiators are Irgacure-2959 (I-2959) and lithium arylphosphanate (LAP), both of which generate free radicals by photocleavage. I-2959 remains a popular choice among users, but has some drawbacks when compared to LAP. I-2959 is not very water soluble (<0.5 wt%), and has low molar absorption at 365 nm ($\epsilon < 10 \text{ M}^{-1} \text{ cm}^{-1}$), the wavelength usually used to excite UV-activated photoinitiators. As discussed previously, the lower molar absorption greatly impacts the polymerization rate, so that large amounts of photoinitiator or long, strong exposures must be used (eqn (1.1)). Due to its low solubility, I-2959 is usually incorporated at high concentrations into solely synthetic polymer systems with non-water based solvents, or exposed for long periods of time in natural polymer systems without encapsulated cells. LAP, on the other hand, has a high absorption at 365 nm ($\epsilon \approx 200 \text{ M}^{-1} \text{ cm}^{-1}$) and is very water soluble (at least 8.5 wt%), making it more conducive for work with natural, composite natural/synthetic polymers solutions, and encapsulated cell solutions. A comparison by Fairbanks *et al.* between I-2959 and LAP at 365 nm with comparable intensities and initiator concentrations found that the time to gelation was nearly an order of magnitude higher using LAP as the photoinitiator. Although commercially available, LAP can also be synthesized in-house nearly overnight, making it a reasonable option for users.^{16,18}

1.3 Biomaterial Choice for Microstereolithography

One of the most common materials for representing the extracellular matrix (ECM) is the hydrogel, a solid material consisting of cross-linked polymer strands that can be tuned for stiffness, adhesiveness, and cell signaling potential. Hydrogel scaffolds can be printed using natural polymers, synthetic polymers, combinations thereof, and combinations with other materials such as carbon nanotubes or nanoparticles.³ Natural polymers are often more cell compatible, can have cell-controlled degradability, low immune response, and are good choices for studies involving direct cell encapsulation within the gel. However, synthetic polymers offer a greater amount of control over the scaffold shape, have better batch consistency, a greater range of mechanical properties and are much more robust overtime, making them good options for seeded cell studies.^{2,3} By combining synthetic and natural polymers or materials in specific concentrations, scaffolds can be generated that are both cell compatible and have the appropriate physical, chemical and mechanical properties to support tissue growth in a biomimetic fashion.

1.3.1 Natural Polymers

There are several common natural polymers used for scaffolds, most common of which are hyaluronic (HA) and gelatin derivatives. Hyaluronic acid, or hyaluronan, is a polysaccharide that plays a key structural role within cartilage, as well as promoting cell motility and differentiation, and naturally

degrades over time.¹⁹ Gelatin, or denatured collagen, is a major ECM component that is biocompatible and biodegrades overtime. Although denatured, it maintains some cell binding moieties including arginine-glycine-aspartic acid (RGD) sequences, which promote cell attachment, and target sequences of matrix metalloproteinase (MMP) for cell remodeling.^{3,14} One popular derivative for these materials is the functionalization of methacrylate groups to form methacrylated hyaluronic acid (meHA) and gelatin methacrylate (GelMA).^{3,14} These natural polymers undergo radical polymerization at rather mild conditions; room temperature, neutral pH, and aqueous environments that are favorable for stereolithography with cell encapsulation (*i.e.*, 3D bioprinting).³

GelMA has been used fairly prominently by itself and with encapsulated cells to form complex and biomimetic structures.^{20–22} Although it can physically crosslink at low temperatures or high concentrations, a GelMA solution exposed to UV light will covalently crosslink in a more stable fashion, and in user-defined shapes. As GelMA is synthesized in-house, either adding different amounts of methacrylic anhydride to the reaction, or changing the pH to make substitution more or less favorable can modulate the amount of methacrylation on the polymer. This in turn impacts the GelMA properties. In polymer scaffolds with more methacrylation groups, there is a greater degree of cross-linking, and thus smaller pore sizes and less swelling. Similarly, fewer methacrylation groups results in greater pore sizes and greater swelling. In one study, the average pore sizes of the gel, after freeze drying, were 50, 30, and 25 μm for 49.8%, 64.8%, and 73.2% substitution, respectively. In addition, the compressive modulus of the hydrogels increased with substitution rate, ranging from 2 kPa, to 3.2 kPa, to 4.6 kPa for 49.8%, 64.8%, and 73.2%, respectively.^{3,23} Thus, the user can modulate the mechanical properties of the gel simply by changing the amount of methacrylic anhydride within the reaction. Moreover, the user can modulate stiffness and pore size using exposure time and strength (Figure 1.2).²⁴

1.3.2 Synthetic Polymers

Although not as conducive to cells, synthetic polymers are a favorable option when the user wants to gain additional printing control or durability. Synthetic materials have a narrower range of molecular weights, resulting in greater batch-to-batch printing consistency. In addition, the length and shape of the polymer can be chosen specifically to modulate porosity and stiffness of the scaffold. For example, a short polymer with two functional groups on either end will form smaller pores and be stiffer than a very long chain polymer with two functional groups on either end. Synthetic polymers can also be purchased in more complex, branched shapes, such as “stars”, to increase cross-linking density.^{2,25} One of the most common polymers is the commercially available hydrophilic polymer polyethylene glycol (PEG), which can be functionalized with different reactant groups to make it sensitive to free-radical polymerization. As with GelMA and meHA, a

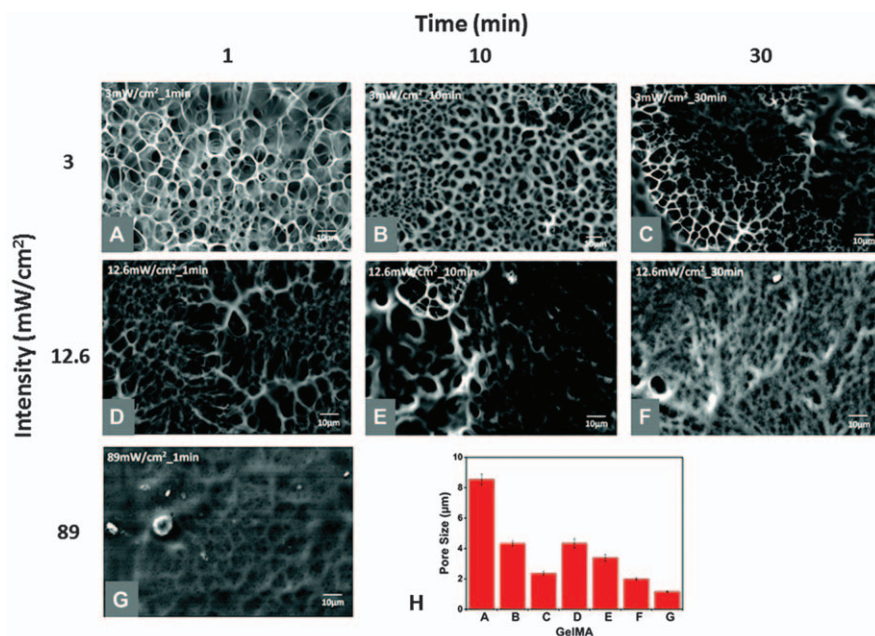


Figure 1.2 (A–G) SEM images of GelMA samples prepared using different UV cross-linking intensities and exposure times. (H) Average pore size (measured from the SEM images) vs. the UV cross-linking intensity and exposure time. Error bars represent standard error; values for A–G are $\pm 0.36 \mu\text{m}$, $\pm 0.19 \mu\text{m}$, $\pm 0.14 \mu\text{m}$, $\pm 0.3 \mu\text{m}$, $\pm 0.22 \mu\text{m}$, $\pm 0.09 \mu\text{m}$, $\pm 0.05 \mu\text{m}$, respectively. Reproduced from ref. 24 with permission from The Royal Society of Chemistry.

common choice is to functionalize acrylate groups onto either end of a PEG chain to form PEG-diacrylate (PEGDA).¹⁴ As PEG scaffolds contain no natural bind moieties, either adhesive peptide sequences such as arginine–glycine–aspartate–serine (RGDS) and tyrosine–iso-leucine–glycine–serine–arginine (YIGSR) or adhesive proteins such as fibronectin or laminin must be grafted on to aid in cell growth.²⁶ Other synthetic polymers for photopolymerization include poly(*N*-isopropylacrylamide) (PNIAAm), poly(acrylic acid) (PAA), polymethylmethacrylate (PMMA), polyacrylamide (PAAm), and poly(dimethylaminoethylmethacrylate) hydrochloride (PDMAEM).²

1.3.3 Composite Materials

Using composite solutions of natural polymers and synthetic polymers, or even natural polymers and various synthetic materials, can provide improved mechanical properties, structures, and even add additional capabilities to the scaffolds.

Most often, synthetic materials are used to add additional stability and durability to natural polymers. A common application can be to add a PEG

polymer to a GelMA or HA polymer to create a stronger, interpenetrating network (IPN).²⁷ Moreover, synthetic materials can also be functionalized with groups for controlled degradation. For example, thiol-ene click-chemistry reactions have been employed for polymerizing binding moieties such as the RGD motif to a PEG scaffold, allowing cells to attach and locally degrade the gel, resulting in a spreading morphology.^{28,29} In addition, recent studies exploring various click chemistries have been able to incorporate photodegradable groups onto PEG, making the possibility for more complex structures even greater.^{30,31} According to studies, click reactions require less photoinitiator and are less oxygen sensitive, leading to more consistent print batches. In addition, “-ene” molecules do not react with itself, so by functionalizing a natural material with norbornene and a synthetic material with a thiol group, the user can be sure they have a well-mixed scaffold.³² Thiol-ene reactions are often referred to as step-growth polymerizations (Section 1.2.1), although the polymerization results from free-radical reactions, as the rate kinetics resemble that of step-growth (Figure 1.1).⁶ Norbornene in particular has fast polymerization due to ring strain relief, making it an excellent choice for fast printing.⁷

Beyond PEG-GelMA and PEG-HA polymer networks, various nanoparticles have been incorporated into GelMA systems to enhance mechanical properties, including carbon nanotubes (CNT), graphene oxide (GO), inorganic nanoparticles, and other biopolymers.

Although increasing the GelMA concentration in a pre-hydrogel solution will lead to enhanced mechanical stiffness (Section 1.3.1), the resulting increase in cross-linking impacts degradability, porosity, cell spreading, and cell growth. Similar to synthetic polymers, synthetic nanomaterials can increase the mechanical stiffness of the scaffolds, but they have the added benefit of not compromising the basic cross-linking structure. For example, Shin *et al.* have carried out studies exploring both the addition of CNTs and GO to GelMA pre-hydrogel solutions.^{33,34} When incorporating CNTs, the nanoparticles were first coated with GelMA (for better dispersion), mixed into a GelMA pre-hydrogel solution, and then polymerized. This resulted in an increase in the elastic modulus from 15 kPa in a 5% GelMA hydrogel to ~60 kPa in a 5% GelMA – 0.5 mg mL⁻¹ CNT hydrogel for the same curing time. Moreover, the gels exhibited a higher toughness and stronger tensile strength when the CNTs were incorporated. The solutions were also shown to be printable and supportive to encapsulated cell culture.³³ The same group followed up this study by incorporating GO into the pre-hydrogel solution. The experiment was expanded to a variety of UV curing times; it was found that the compressive modulus range for 5% GelMA was 5 to 9 kPa and the range of 5% GelMA – (0–2.0) mg mL⁻¹ GO was 4 to 24 kPa. In addition, the GelMA-GO gels exhibited enhanced electrical conductivity, and cells were found to proliferate more quickly on the GelMA-GO scaffolds, possibly due to stronger cell adhesion.³⁴ As nanotechnology continues to advance, the availability and innovation of printable materials advances as well.

1.4 Scanning-based Microstereolithography

Photopolymerization can only be triggered by light intensity over a certain threshold. The idea of scanning-based microstereolithography is to use a lens to focus the beam, so that the intensity is over the polymerization threshold at only a specific focal point. A desired 3D structure can be made by scanning the focal point through the volume of the whole design. Clearly, this is a serial process because 3D fabrication relies on point scanning, which limits the throughput of this method. Scanning-based microstereolithography is also referred to as direct laser writing.

1.4.1 Single-photon Polymerization Scanning-based Methods

Typical single-photon scanning-based stereolithography uses a microscopic objective lens to focus the UV laser beam (Figure 1.3). The photocurable prepolymer solution is loaded in a vat, a focused UV laser then induces photopolymerization at its focal point. The motion of the fabricated structure is controlled by a 3D computer numeric control platform, and the on/off state of the laser beam is manipulated by a computer-controlled shutter. Hence, photopolymerization can happen only at desired positions. During fabrication, the platform first moves to a position which is slightly under the liquid surface, then translates in a 2D horizontal plane, thus forming a thin layer of 2D solid structure made from the liquid solution. Then the platform brings the structure down a certain distance in the z-direction, allowing a thin layer of unpolymerized solution to cover the top of the polymerized structure. Another round of 2D translational scanning follows, and a new layer of 2D structure is stacked on top of the previous layer. Therefore, a 3D structure can be fabricated by this layer-by-layer scanning process.³⁵

An alternative way of scanning is to introduce a 2D galvo scanning mirror to the system. In this setup, the platform can only move vertically, and the

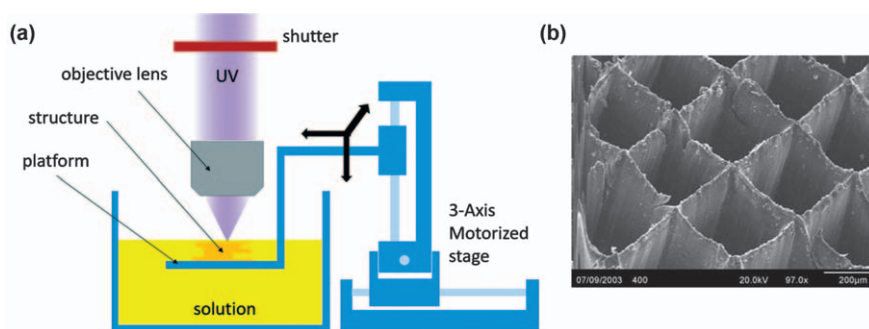


Figure 1.3 A typical single-photon stereolithography setup (a), and scanning electron microscope image of a structure fabricated by single-photon stereolithography (b). Scale bar: 200 microns.

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2D horizontal scanning is performed by using galvo scanning mirrors to steer the focal point of the laser. Generally, galvo mirrors has a higher scanning speed but smaller scanning range than computer numeric control platform.

The resolution of single-photon stereolithography is on the micron scale, which is ideal for making biomimetic scaffolds.³⁶ It can be used for fabrication of cell encapsulation structures, tissue engineering scaffolds, implantation parts, drug delivery devices, and so on.^{35,37–40}

1.4.2 Two-photon Polymerization Scanning-based Methods

Two-photon absorption is a physical process where a molecule absorbs two photons simultaneously to transition to a higher energy electronic state. The energy difference between the two states is equal to the sum of the two photons. That means that in a two-photon absorption scenario, the photon wavelength is two times as long as in a one-photon absorption scenario. Two-photon polymerization refers to photopolymerization initiated by two-photon absorption, which can happen to some photoinitiators that have a large two-photon absorption cross section (molecular probability of two-photon absorption event).^{41–43} For example, bis(2,4,6-trimethylbenzoyl)-phenylphosphineoxide (product name: Irgacure 819) can initiate photopolymerization with irradiation under 440 nm wavelength. It can also initiate photopolymerization with 800 nm ultrafast laser, where the sum of the energy of the two photons is the same as the energy of a 400 nm photon.

Table 1.1 lists the two-photon absorption cross section of common photoinitiators.⁴¹ Z-scan and WLC-2PA are two different measuring methods. $\lambda_{\max}(\text{SPA})$ and $\lambda_{\max}(\text{2PA})$ denotes the wavelengths (in nm) of absorption

Table 1.1 Two-photon absorption cross section of common photoinitiators measured with two different methods. Adapted from ref. 41 with permission from Elsevier, Copyright 2004.

photoinitiator	$\lambda_{\max}(\text{SPA})$	Z-scan		WLC-2PA	
		$\lambda_{\max}(\text{2PA})$	σ_{2PA}	$\lambda_{\max}(\text{2PA})$	σ_{2PA}
Irgacure 184	246	530	23	500	<20
Irgacure 261	242	530	<20	500	<20
Irgacure 369	324	670	7	636	27
Irgacure 651	254	530	28	500	<20
Iagacure 754	253	530	21	500	10
Irgacure 819	295	600	<4	600	<5
Irgacure 907	306	600	4	600	<5
Irgacure OXE01	328	660	31	660	38
Darocure TPO	299	600	<4	600	<5
Darocure MBF	255	530	27	500	<20
Darocure 1173	244	530	<20	200	<20
CD1012	247	530	16	546	14
ITX	382	760	5	754	4
DPABz	390	780	100	776	120

peaks for single-photon absorption and two-photon absorption, respectively. σ_{2PA} is the measured two-photon absorption cross section, the unit is in Goppert Mayer unit (GM), where $1 \text{ GM} = 10^{-50} \text{ cm}^4 \text{ s photon}^{-1}$.

Two-photon polymerization requires extremely high power density. Thus an ultrafast pulsed laser should be used, and the laser beam should be tightly focused by a high numerical aperture objective lens. Figure 1.4 shows a typical two-photon stereolithography setup. A Ti sapphire pulsed laser (wavelength = 800 nm, pulse width = 100 fs) and a high numerical aperture objective (NA = 1.3) are used. The working average power is 5 mW, and the writing speed is 50 microns per second.^{44,45} Galvo scanning mirrors can also be used in two-photon stereolithography to enhance scanning speed.

The light intensity profile at the focal spot is a Gaussian function, and the focal spot size, *i.e.* the full-width-at-half-maximum (FWHM) of the Gaussian function, can be estimated by $D = 0.61\lambda/\text{NA}$. For an 800 nm laser and NA 1.3 objective, the focal spot size is around 375 nm. Due to the nonlinear nature of two-photon absorption, the two-photon polymerization initiation probability is proportional to the square of the light intensity. Hence, the FWHM of the two-photon polymerization initiation probability profile is narrower than that of light intensity profile. Therefore, the line width of two-photon stereolithography is smaller than the focal spot size.⁴⁶ Sub-micro resolution down to 100 nm can be achieved by two-photon stereolithography, which is much finer than single-photon stereolithography.⁴⁷

An important feature of two-photon stereolithography is that it can write inside the prepolymer solution, instead of being limited to surface. The prepolymer solution, which consists of monomer and photoinitiator, has a

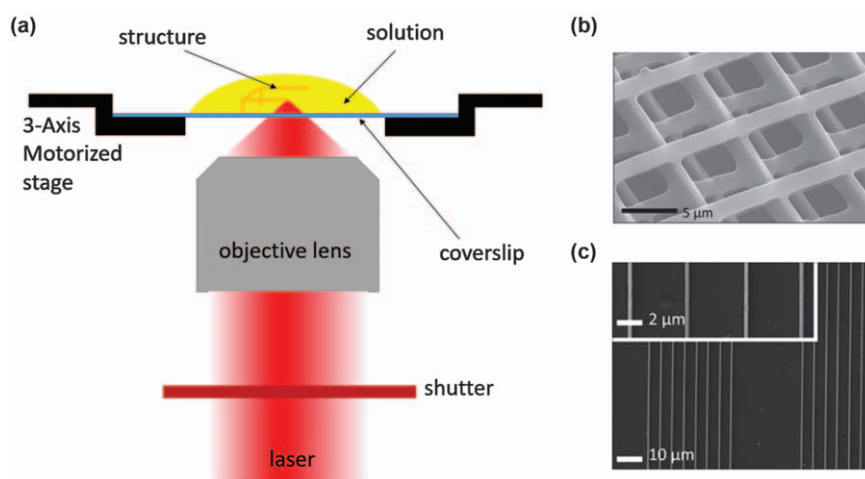


Figure 1.4 A typical two-photon stereolithography setup (a), and scanning electron microscope images of the structures fabricated by two-photon stereolithography (b and c).

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high absorbance in UV-range but very low absorbance in the visible and near-infrared (IR) range. As a result, in single-photon stereolithography, UV light can only polymerize the surface of the prepolymer solution; in two-photon stereolithography, the near-IR light can penetrate into the prepolymer solution and induce two-photon polymerization inside the solution. Compared to single-photon stereolithography, there are no movable parts, such as a motorized platform, invading in the solution. Therefore, solid state prepolymer solutions such as soft-baked negative photoresists are also compatible with this technique.

Research on two-photon absorption for stereolithography was first published in 1997.⁴⁸ Its free-form fabrication capability and sub-microscale resolution have attracted much research attention in the following decades. This technique has been reported to be used in the research of photonic crystals, photonic metamaterials, mechanical metamaterials, micro-machines, microrobots, protein microstructure, cell behavior study, tissue engineering, and so on.^{49–55}

1.5 Projection-based Microstereolithography

Projection-based microstereolithography is a parallel process. Instead of point scanning, this method projects an image plane in the prepolymer. By scanning this plane in the z-direction, a 3D structure can be fabricated.

1.5.1 Digital Light Processing

Traditional 2D photolithography is the most important technology in the modern semiconductor industry. A typical projection-based exposure system for photolithography contains a UV light source, photomask, lens, and a photoresist. The UV light is patterned by the photomask and is then projected onto the photoresist by a lens.

Recent advances of digital light processing (DLP) devices have allowed maskless photolithography. These devices include liquid crystal display (LCD) and digital micromirror devices (DMDs).

A LCD DLP device is an electrophotonic device. Typical transmitting LCD devices have a multilayer structure: one polarizer layer, one electrode array layer, one liquid crystal layer, one common electrode layer, and one polarizer layer (Figure 1.5(a)). The two polarizers has perpendicular polarizing axis; thus, the device has no transparency when no voltage applied. The liquid crystal has birefringence property. When voltage is applied, the liquid crystal is reoriented, and its extraordinary refractive index is changed. Therefore, the liquid crystal can rotate the polarization axis of a linear polarized light. At certain voltage, the polarization direction of light can be rotated by 90 degrees, so the light can pass through the second polarizer. Thus, by tuning the voltage on each pixel of the electrode array, the transmittance of each pixel on the LCD can be tuned, and light can be patterned.

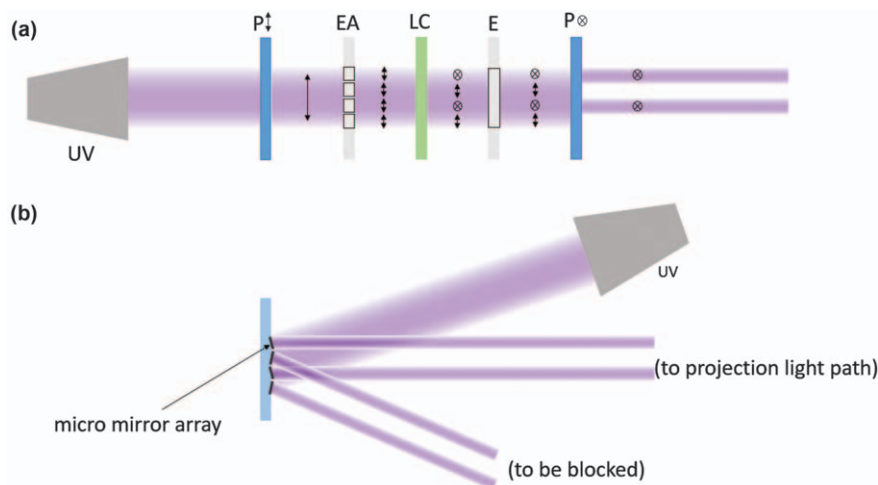


Figure 1.5 Transmitting LCD DLP device (a) and reflecting DMD DLP device (b). UV: ultraviolet light source; P: polarizer; E: electrode; EA: electrode array; LC: liquid crystal. The arrows in (a) denote the polarizations.

DMD DLP device is a micro-electro-mechanical system (MEMS), which consists of millions of micro mirrors that can flip to two different angles (Figure 1.5(b)). By flipping the mirrors, the incoming light can either be directed into the projection light path, or be deflected out of the projection light path. By individually controlling each micro mirror on the DMD chip, a desired pattern can be projected onto the photosensitive polymers.

Inspired by maskless photolithography, microstereolithography methods using LCD and DMD were invented in the 1990s and 2000s, respectively.^{56,57} Compared to the scanning-based microstereolithography, these projection-based methods provide extremely fast fabrication speeds since it is a parallel process. A complex 3D structure on the millimeter scale can be fabricated in mere seconds. This high throughput feature is very attractive for mass production in industry. Furthermore, fabrication of cell-laden biomaterials become much easier by this method, because the cells only have to endure the out-of-incubator environment for a very short time.^{20,58}

1.5.2 Liquid–Air Interface Polymerization Setup

Projection-based microstereolithography setup can be categorized into two classes, based on the position where photopolymerization takes place.⁵⁹

In the first class of setup, photopolymerization happens at liquid–air interface.^{60–63} The prepolymer solution is loaded in a vat (Figure 1.6(a)). The UV light is modulated by a DLP device and projected from the top of the vat. Hence, photopolymerization happens at the liquid–air interface. During fabrication, a motorized platform first moves to a position slightly under the liquid. A pattern is then loaded on the DLP device and projected onto the

prepolymer solution, fabricating a 2D structure in one exposure. The platform then brings down the structure to allow a thin layer of unpolymerized liquid to cover the fabricated structure, and the DLP device loads the mask for the next layer. It is then followed by another exposure to create another layer of 2D structure. By this layer-by-layer exposure process, a 3D structure is fabricated.

In order to achieve a high quality fabrication, the liquid–air interface should maintain good flatness. Therefore, the meniscus caused by surface tension and any ripples caused by motion should be avoided after the platform moves down to get ready for the next layer. There are two ways to make a flat surface, as outlined below.

One, instead of directly moving to the desired z position, the platform first moves to a z position which is much lower than the target position, then rises back to the target position. This roundabout motion ensures the unpolymerized liquid can efficiently cover the polymerized structure. The liquid will calm down after a few seconds, making a flat surface for the next exposure.

Another method is to use a recoating blade. The platform directly moves to the target position, then the recoating blade skims through the liquid surface to help making a thin layer of unpolymerized liquid on top of the fabricated structure.

Both ways introduce a time interval between two exposures; therefore, slow down the whole fabrication process, and also introduce visible “interfaces” between layers due to the discrete motion and exposure.⁶³ These interfaces are less obvious than interfaces produced by inkjet-based and extrusion-based 3D printing, yet it could still degrade the structural integrity.

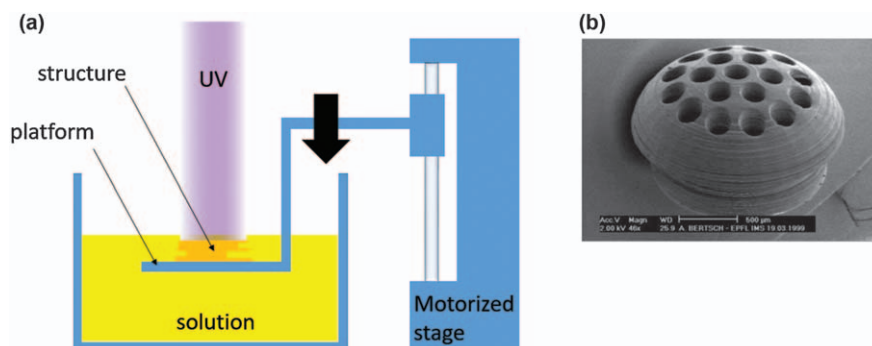


Figure 1.6 (a) A typical setup of projection-based microstereolithography, where the patterned UV light comes from the top and polymerization takes place at liquid–air interface. The platform travels down in a discrete manner during fabrication. (b) Scanning electron microscope image of a fabricated structure. Scale bar in (b) is 500 microns. The interfaces between layers are visible on its surface.

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1.5.3 Liquid–substrate Polymerization Setup

In the second class of setup, photopolymerization takes place at the liquid–substrate interface.^{64,65} The prepolymer solution is loaded into a vat (Figure 1.7(a)).⁶⁶ The UV light is modulated by the DLP device and projected from the bottom of the vat. A transparent anti-adhesion substrate is installed at the bottom of the vat. During fabrication, the platform first moves to a position very close to the anti-adhesion substrate. After exposure, the polymerized structure fills the space between the platform and the anti-adhesion substrate, and adheres to the platform. The motorized platform then moves up to allow a thin layer of unpolymerized liquid to flow into the space above the substrate, and the mask of the next layer is loaded on the DLP device. A second layer of structure can then be fabricated by another exposure. Therefore, a new layer of polymer is fabricated beneath the structure, and eventually a 3D structure is printed in a continuous fashion.

Since the substrate helps to maintain good surface flatness, there is no time interval required between motion and exposure. Hence, the platform motion and UV exposure can both be performed in a continuous manner instead of a layer-by-layer manner as described above. Thus, the fabrication time is significantly reduced. Furthermore, the “interface” between layers is eliminated, resulting in a smooth and layerless surface.

A typical anti-adhesion substrate can be a polydimethylsiloxane (PDMS) membrane and hydrophobic molecular layer coated glass, such as silane.

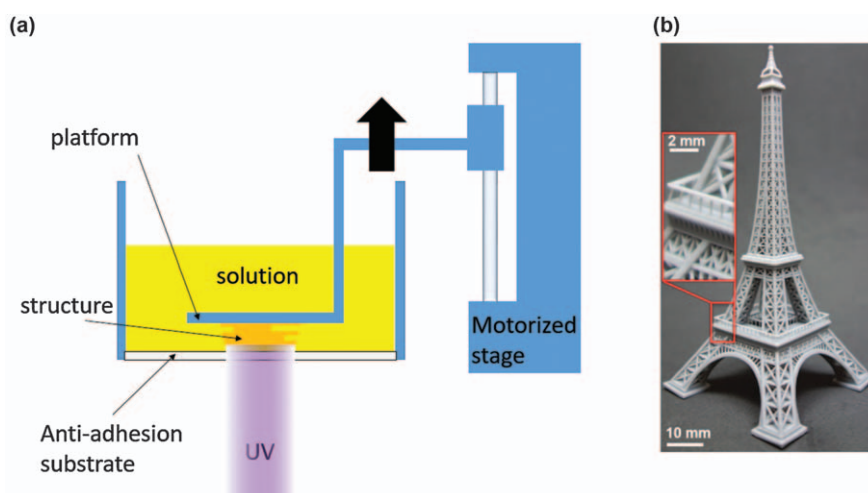


Figure 1.7 (a) A typical setup of projection-based microstereolithography, where the patterned UV light comes from the bottom and polymerization takes place at liquid–substrate interface. The platform travels up continuously during fabrication. (b) Scanning electron microscope image of a fabricated structure. The surface of (b) is smooth. Adapted from ref. 66 with permission from American Chemical Society, Copyright 2016.

An oxygen permeable window is also reported as anti-adhesion substrate, which allows excessive concentration of oxygen to deplete free radicals and impede polymerization in a thin layer near the substrate.⁶⁴

Projection-based microstereolithography is able to fabricate a complex 3D structure with micron scale resolution in several seconds to several minutes. Thus, intense research interest has focused on this technique. It is finding extensive applications in microrobots, electronic devices, imaging phantoms, tissue engineering, medical implantation, and so on.^{20,67–70}

1.6 Summary and Outlook

Microstereolithography is a promising technology for free-form 3D fabrication in micrometer and sub-micrometer scale. A wide variety of materials, especially biomaterials, are compatible to this method.

Scanning-based microstereolithography is a time consuming 3D fabrication method due to its point scanning process. Yet this method provides microscale fabrication resolution. Two-photon scanning-based stereolithography can achieve 100 nm resolution, and even finer resolution can be achieved by super-resolution stereolithography (tens of nm).

Projection-based microstereolithography has developed into the most attractive microstereolithography method. This method offers a remarkable fast fabrication speed (within a few seconds to minutes) as well as fine resolution (lateral resolution at micrometer scale), which are both better than traditional inkjet-based and extrusion-based 3D printing. Although the utilization of UV light may be considered harmful to cells, the fast fabrication speed can minimize the exposure time. Thus cell-laden materials are also compatible to this method.

In light of the rapid evolution of microstereolithography technology, it is reasonable to believe that faster fabrication speed, finer resolution and more compatible materials can be achieved in the future.

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CHAPTER 2

*Extrusion-based Bioprinting*MITCHELL KUSS^{a,b} AND BIN DUAN^{*a,b,c}

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2.1 Extrusion-based Bioprinting**2.1.1 Bioprinting**

Bioprinting is an additive manufacturing technique in which there is the automated deposition of cells or other biological materials into a predetermined pattern, using a layer-by-layer process, in order to create three-dimensional (3D) tissue constructs.¹ Bioprinting techniques were adapted from traditional 3D printing methods in order to incorporate cells without causing harm to them. A general goal of bioprinting is to deliver a construct with a defined architecture that can mimic native tissue in both form and function. This involves using the right materials to mimic the properties of the tissue, along with the right cells and biologics that will allow for growth and integration with the body. A bioink is the material that contains the cells and/or biologic that is used in the bioprinting process.² Depending on the type of tissue, and its associated properties, that is desired to be printed, the bioink used can be made from a number of different materials. To create the desired shapes for the constructs, bioprinting modalities can use

computer-aided design (CAD) software to create standard or custom designs, or medical imaging techniques, such as magnetic resonance imaging (MRI) and computed tomography (CT), to create personalized designs.

2.1.1.1 Benefits of Bioprinting

Bioprinting has its benefits when compared to traditional tissue engineering techniques. It is difficult for standard techniques to create scaffolds for tissues or organs that are complex in shape, makeup, and/or function.³ Some tissues and organs have a heterogeneous composition and are difficult to create with standard techniques. Bioprinting helps to solve this problem by allowing the deposition of material in precise locations, which enables the controllability of size, shape, pores, and others.⁴ Some techniques create toxic environments for cells,^{5,6} but bioprinting allows scaffold creation without the use of harsh chemicals or processes.

2.1.1.2 Types of Bioprinting

There are three main types of bioprinting. They are inkjet, light/laser-assisted, and extrusion-based.^{7,8} Inkjet bioprinting is a technique that has multiple types of devices that use heat to create tiny bioink droplets, use piezoelectric energy to create an ejection of small droplets, or are laser-assisted to cause vaporization of the droplets.⁹ These droplets build up layer by layer to create a 3D structure. This method can have a high resolution but can be plagued by low cell viability,^{10,11} a long printing time,^{12,13} and a low number of bioink options, because they cannot use highly viscous materials.^{14–16}

One method of light/laser-assisted bioprinting uses a laser to vaporize the solution and eject the bioink substances onto the substrate.¹⁷ A donor layer, on top of a bioink layer, absorbs energy from the laser and creates a bubble that propels the bioink out.¹⁸ Another method is light stereolithography, using light or a laser.¹⁹ These methods have high resolution and cause low mechanical stress on cells, but they require expensive equipment, can be inefficient, have a limited number of bioinks, and can cause failure of biological materials.^{17,18} Extrusion-based bioprinting (EBB) is a form of bioprinting that uses force to extrude bioinks out of a nozzle and onto a substrate in order to create a 3D object.^{1,20} It is a fairly simple concept that is very effective. This method can use a wide range of materials, has a high printing speed, and is relatively inexpensive, but it can cause mechanical stress on the cells and has a relatively lower resolution than other bioprinting methods.^{21,22} EBB seems to be a promising bioprinting method for tissue and organ engineering. It is rapidly advancing in its applications and abilities.

2.1.1.3 Bioinks

Bioinks are the biomaterials with cells and/or biologics that are used in the bioprinting process.²³ The bioink choice is important because it will

determine the effectiveness of the scaffold that is bioprinted, and each type of bioprinting will only function correctly when using a certain type of bioink. Bioinks can provide a supportive local environment and protection during the printing process.²⁴ The proper bioink for a certain job will possess the correct cells and biologics, viscosity for bioprinting, crosslinking or reactivity characteristics, mechanical properties, and reactivity in the body.²⁵ There is always a need for new and better bioink combinations in order to increase printability and cell function.²⁴ The main types of bioinks are hydrogels and decellularized extracellular matrix.^{23,26}

2.1.1.3.1 Hydrogels. Hydrogels are crosslinked polymeric substances that are able to take up and store large amounts of water.²⁵ They are the most popular of the bioinks due to the large diversity of materials, with many varying physical and biological properties, that can be used to create them. The hydrogels do a great job of supporting cell viability during encapsulation because they can somewhat mimic native tissue, as they are highly hydrated, mechanically tunable, and some contain extracellular matrix (ECM)-like properties. Hydrogels are also permeable to water and nutrients, which allows for the movement of key compounds that promote cell growth and development. Different hydrogels can be used for EBB, inkjet, or light/laser-based bioprinting.²⁷ The properties of hydrogels can be tuned for the types of cells and the type of bioprinting desired by changing the material type and the concentrations of these materials. There are some varieties that can be turned into droplets, some that are photocrosslinkable, and some that can be extruded then crosslinked.² The biological properties, as well as the mechanical and chemical properties, can be adjusted to the desired characteristics. Hydrogels can also be made of natural or synthetic polymers, depending on the desired properties of the hydrogel. Some natural materials commonly used to create hydrogels for bioprinting are agarose,²⁸ alginate,²⁹ chitosan,³⁰ collagen I,³¹ fibrin,³² hyaluronic acid (HA),³³ and gelatin.³⁴ Some synthetic polymers commonly used to create hydrogels are methacrylated gelatin (Me-Gel),³⁵ Pluronic[®] F-127,³⁶ and poly(ethylene glycol) (PEG).³⁷ Of course, there are other materials that can be used to create hydrogels, and more are being tested and created all the time. The choice of material used to make the hydrogel depends on the cell type, bioprinting technique, and desired mechanical properties.

Hydrogels must be crosslinked to go from their precursor state to full hydrogels with mechanical stiffness. Some methods of physical crosslinking for hydrogels are ionic crosslinking, temperature dependent crosslinking, and self-assembly. The physical methods of crosslinking generally have a low risk of gaining cell toxicity in the hydrogel, and the chemical methods of hydrogel crosslinking are more likely to be toxic to cells,³⁸ but create better mechanical stability due to covalent bonding. The chemical crosslinking comes from the main methods of photocrosslinking or adding a solution to the hydrogel in order to cause a reaction that crosslinks it. Another form of

crosslinking is enzymatic crosslinking. The type and extent of crosslinking are highly dependent on the material used and the desired mechanical properties of the hydrogel.

2.1.1.3.2 Decellularized Extracellular Matrix Bioinks. Hydrogels may not perfectly provide the local environment that best supports cell growth and function in a printed construct, so ECM bioinks can be used. ECM is the local environment surrounding cells that promotes their function, growth, and development.³⁹ To isolate the ECM, physical, chemical, and enzymatic reactions are used to remove the cells from the ECM while leaving all of the molecules that make up the ECM.⁴⁰ The decellularized ECM is further solubilized by crushing the ECM into a powder and mixing it with a buffer solution, which creates a gel-like material that can be bioprinted.⁴¹ Decellularized ECM is a good material for bioprinting because it is gel-like and has high viability for cell growth and function due to the fact that it is made of the cells' naturally-produced local environment. These bioinks can have issues with residual toxicity, may need a frame to support them, and it can be difficult to get large amounts of them.²¹

2.1.2 EBB Systems

In general, EBB uses a physical force to push a bioink out of a nozzle and deposit it in a layer-by-layer manner to create a predesigned 3-dimensional shape. This is done by loading a cell-laden bioink into a reservoir with a nozzle on the end of it then using an automated system to use a form of pressure to extrude the bioink through the nozzle in a pattern predetermined by CAD software. The bioink is then crosslinked in order to create a mechanically stable scaffold of the desired shape.

2.1.2.1 Design of EBB Systems

EBB is done using a combination of a fluid-dispensing system with an automated robotic movement system.⁴² With EBB, a bioink is loaded into a metal or plastic syringe that can be temperature controlled. Depending on the need, an EBB machine can have multiple syringes available for use in one bioprinting session.^{43,44} The temperature of each syringe is computer controlled to ensure precise control, is based on the needs of the bioink, and should be separately controlled to allow for the adjustment to the optimum temperature for different bioink recipes that may be used at the same time. The syringes are attached to nozzles that are generally micro-sized. The smaller the nozzle size, the better the resolution, but not all bioinks can be printed through smaller nozzles, due to their viscosities or particles clogging the nozzle. To extrude the bioinks out of the nozzle, the syringes are attached on the other end to a pressure control system that is controlled by a computer. There are multiple types of pressure control methods, including pneumatic, piston-driven, and screw-driven systems.^{1,45} As the pressure

system extrudes the bioink out of the nozzle, there is very precise, computer-controlled movement along the x , y , and z -axes that allows the bioink to be printed in a very specific location.⁴⁶ This movement can be performed by either the syringe or the platform moving along a three axis system. The precise movement control along the three axes allows for the specific shape to be printed and built in a layer-by-layer manner on top of the previous layer. After printing, crosslinking or curing is performed to ensure that the bioink becomes mechanically stable and holds its printed shape.

2.1.2.2 Functioning of EBB Systems

There are many pieces and processes that work together in order for an EBB system to function properly. It begins with the design of the object that is to be printed, then goes to the software for programming the movement and use of the system, the movement system, the nozzle, the extrusion system, and finally the crosslinking method. All of these things need to work together for the system to fully function.

2.1.2.2.1 Design of Product and Programming. The designing of the object to be printed is crucial for the bioprinting to work. If the design is wrong, then the object might not function as intended or fit where it needs to fit. The design can be created using CAD software. By using CAD software, a custom design can be created, and if done right, design changes can be made easily as needed. There are free versions of CAD software systems, like FreeCAD and Fusion 360, as well as more advanced ones that require subscriptions, like Solidworks and AutoCAD.

Another way to get a design for use in EBB is by using medical imaging, like CT, MRI, or other imaging modalities.⁴⁷ Using these medical images allows the bioprinting of personalized products. These personalized products can be just about anything that can be imaged, such as defects and partial or full replacements of a body part. Whether using CAD software or medical imaging to create the design to be bioprinted, the design needs to be converted into a file type that is readable by the software being used to run the bioprinter.

If a commercial EBB system is being used, it will come with software that runs the machine. A common file type that this software will use for the design is an STL file. This is a common file type for most design software to be able to convert files to. The software used to run the bioprinters will vary, but they all will have the ability to read in the design file and run the machine to print that design exactly as it was made. They will allow the user to make changes to the printing pattern, which can consist of pore sizes, layer sizes, and which bioinks are printed in which areas. They will also allow the adjustment of printing parameters, including temperature, print speed, and extrusion speed. Adjusting these parameters in the software used to run the machine allows the user to optimize all of the parameters to each specific bioink in order to get the best printing from them.

2.1.2.2.2 Robotic Movement of EBB Systems. The movement of the EBB system is crucial to its success, and is therefore precisely controlled by the automated robotic system. Depending on the machine needs, either the syringes or the platform can be the movable part of the machine. The linear robotic movement is along the x , y , and z -axes, meaning that it can move in all directions on the horizontal plane, as well as move up and down vertically.³ Being able to move in any direction on the horizontal plane allows the bioprinting of complex shapes with precise movements. The movement on the vertical axis allows for the printing in a layer-by-layer fashion. After one layer is finished, either the syringe or the platform will move along the vertical axis at a defined layer height so that the next layer can be printed on top of the previous layer.⁴⁸ As robotic technology advances, more precise and complex movements can be achieved. Robots with 6 degrees of freedom in their movement, such as the BioAssemblyBot, have the same vertical and horizontal placement capabilities as linear robotic movement systems, but they can also move the printing head to be positioned at an angle. This means that this system has control over vertical, horizontal, and angled positioning of the print head, allowing for placement of the head in more complex positions than the standard linear robotic movement system.

2.1.2.3 Mechanisms of Extrusion

The main systems for producing the mechanical force to create pressure on the bioinks for their extrusion in EBB systems are pneumatic, piston-driven, and screw-driven (Figure 2.1).⁴⁹ These might all function differently, but they produce the same effect on the bioink. The goal is to create a mechanical force that applies pressure on the bioink, extruding it out of the nozzle at the other end. They all have their benefits and drawbacks.

2.1.2.3.1 Mechanical Force Extrusion Systems. Piston- and screw-driven extrusion systems are both mechanical force extrusion systems and are

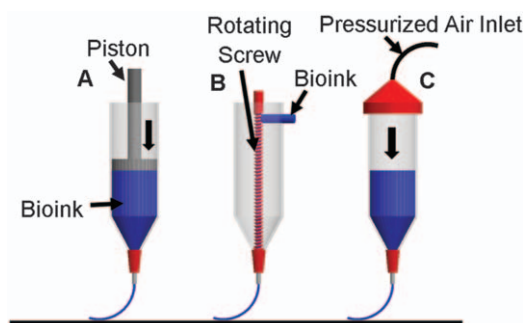


Figure 2.1 Modes of EBB. (A) Piston-driven. (B) Screw-driven. (C) Pneumatic-driven.

very similar to each other in basic principle. A piston-driven system uses a piston to directly apply a linear force through the syringe, thus dispensing the bioink. This extrusion system allows for the close control of the flow rate of the bioink out of the nozzle.⁴² Piston-driven extrusion systems can have good cell viability throughout the extrusion process.

A screw-driven pressure system uses the turning of a screw to force the bioink down the syringe, therefore pushing it out of the nozzle. The screw-driven systems allow for high spatial control compared to the other EBB systems, meaning that they can closely control the dispensing of bioink into the proper areas. They are also good to use when using highly viscous hydrogels because high forces can be produced under controlled extrusion speeds. While the ability to produce a large pressure is helpful for printing viscous materials, a problem that can arise with screw-driven bioprinting is that high pressure drops at the nozzle can cause the rupturing of the cells encapsulated in the bioink. This effect can be managed by using a screw mechanism that is carefully designed for this specific technique and not adapted for use in the system.

In these mechanical forms of extrusion, there are moving parts involved in the deposition systems. Although these parts do well to make controllable deposition possible, they can break down or fail, which could lead to inaccuracy or total loss of use, so they must be maintained.

2.1.2.3.2 Pneumatic Extrusion System. Pneumatic extrusion systems for bioprinting are relatively simple and effective. The pneumatic extrusion system forces pressurized air through the syringe to create a controlled pressure within it, which in turn forces the bioink out of the nozzle at a rate dependent on the viscosity of the bioink and the chosen pressure. There are pneumatic systems that are valve-free or valve-based. The valve-free system is simpler, but the valve-based system can be helpful for high precision needs, due to its ability to create more controlled pressures with pressure pulses. Pneumatic extrusion can be useful for printing a wide range of bioinks with varying viscosities.¹⁸ A problem with this system is that there is little direct control over the flow rate of the bioink out of the nozzle, meaning that the pressure used is constant, and the physical movement of the bioink out of the syringe is not being closely controlled, as in the case of the mechanical extrusion systems. The pneumatic system creates a stress on the encapsulated cells that does not usually cause permanent damage, which allows for higher viability after printing.⁵⁰

All of the mechanisms that can be used in EBB systems have their merits and demerits. It is up to the user to find the one that is right for the intended application, based on desired bioink type and components to use, controllability needed, the resolution, and other factors that affect a successful printing.

2.1.2.4 Nozzle Deposition

An important piece of the EBB system that may be overlooked is the nozzle. The nozzle size controls the size of the filament to be printed, in turn

affecting the resolution and fidelity of a printed design. Having a small nozzle is good for increasing the resolution of the printed design, but not all systems and bioinks will be able to use a small nozzle. If a bioink's viscosity is too high to be extruded through the small nozzle by the extrusion system, then a larger nozzle will need to be used to complete the printing. Another reason for needing to use a larger nozzle is that the small nozzle causes higher shear stress on the cells, which can cause cell death.⁵⁰ A larger nozzle will lower the shear stress, thus allowing more of the cells to survive the printing process. It is ideal to use the smallest nozzle possible to extrude the bioink filament consistently while maintaining the encapsulated cell viability. There are also specialized nozzles that can perform functions other than just general bioink deposition.

2.1.2.4.1 Advanced Nozzle Designs. Designing a nozzle that performs tasks other than just linear deposition can be extremely helpful in applications that require more complicated printing to work properly. Multiple types of advanced nozzles have been created (Figure 2.2). One of the applications that needs a more complicated printing technique is creating hollow channel tubes that can be used for vascularization and other applications. To do this, nozzles with multiple, coaxial extrusion needles have been developed.^{17,51} In these coaxial nozzles, extrusion needles are

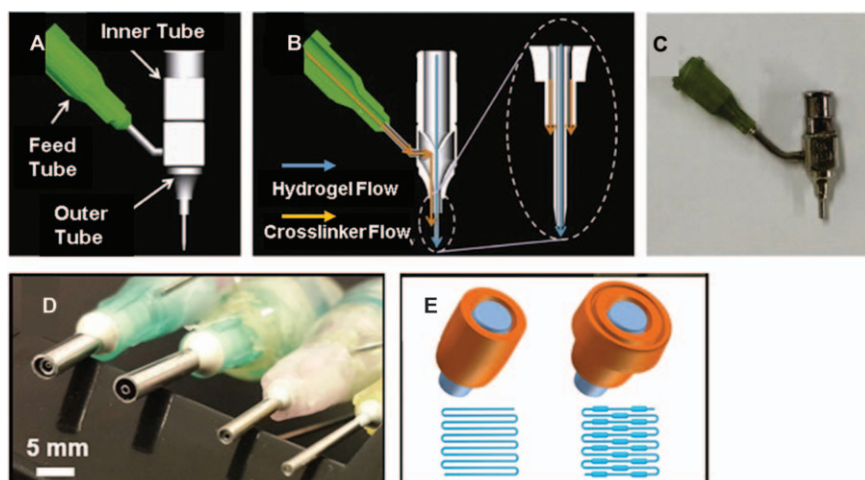


Figure 2.2 Alternative nozzle designs. (A–C) Coaxial nozzle for filament printing. (A) 3D model (B) Cross-sectional view. (C) Physical prototype. (D–E) Multilayered coaxial nozzles with varying sizes for creating hollow tubes. (D) Physical prototypes of coaxial nozzles. (E) Model of different nozzle designs. (A)–(C) reproduced from ref. 51 with permission from Elsevier, Copyright 2013. (D)–(E) reproduced from ref. 52 with permission from Elsevier, Copyright 2016.

combined into one nozzle that allows the flow of a liquid crosslinker through the center needle, while the bioink is extruded through a larger extrusion needle that is around the smaller one. This type of nozzle allows a tube of bioink to be created and crosslinked as it is created, so it retains its tubular shape. There has even been a nozzle developed that has a third extrusion needle, which allows for the crosslinker to flow on both the inside and outside of bioink, crosslinking both sides at the same time.⁵² With these nozzles, the flow rates of the crosslinker and bioink should be able to be controlled independently so that the proper flow ratio to create the desired crosslinking degree that creates the best structure during printing can be achieved.

Nozzles exist that have turns in them.⁵¹ These can be used on a print arm that comes in at an angle so that it can still print vertically. This can allow for multiple print arms to be used simultaneously without running into each other. A curved nozzle could also allow the printing at angles, but it would be harder to control the deposition in the desired shapes. Increased nozzle technology can lead to the increased ability to print more advanced designs, so continuing to create new nozzle designs can be helpful in making more advanced bioprinting capabilities.

2.1.3 Bioinks in Extrusion-based Bioprinting

Probably, the most important piece of an EBB system is the bioink. Bioinks allow the whole system to function as a bioprinter, and they encapsulate the cells and allow them to grow into the intended biological material, while holding the intended shape. They are meant to provide the nutrients and a stable growing environment for the cells. The bioinks also allow for the printability of the cells in specified designs. The proper bioink recipes lead to a high cell viability, the functioning of the EBB bioprinter to print desired shapes, and the effectiveness of the crosslinking method used to make sure the shape is held. Finding the optimum balance of cell-dedicated and structurally-dedicated properties of the bioinks is crucial for the complete functioning of the EBB system. This may mean using a mixture of a variety of bioinks with a variety of properties to complete a process. EBB has specific needs for its bioinks. They must be shear thinning, have low surface tension, have low adhesion, gelate rapidly, and have high shape retention. All of these properties contribute to a bioink printing easily and consistently. The number of bioinks available for EBB use may be limited, but the number is always growing as more and more new materials are being developed.

A benefit of EBB is that it can print bioinks that are usable at temperatures that are safe for the cellular components. Basically, if a bioink can be printed around room temperature or body temperature, then the cells can survive. If a material needed to be at high temperatures to be printed, it would kill the cells or deactivate proteins and nutrients. They can support cells throughout the printing process and into the growth stage. Some are made to support the cells for a short time and others last longer, to provide the structural

support and a microenvironment that mimics the cells' natural microenvironment. Bioinks are developed to be helpful environments for cell growth and function.

2.1.3.1 Hydrogels

The most common bioink category for EBB is hydrogels. The hydrogels used in EBB are generally non-Newtonian fluids because the shear thinning behavior is helpful in EBB. By changing component concentrations, the mechanical properties of the hydrogels can be easily changed, which is a huge benefit when using EBB. This allows for the adjustment of the hydrogel viscosity to allow it to be in the printable range for the EBB system.

2.1.3.1.1 Naturally-derived Hydrogels. Since natural hydrogels are derived from naturally occurring materials that are generally in contact with cells, they usually have favorable ECM-mimicking microenvironments that support cell growth and function. These same hydrogels may also struggle with their mechanical properties before or after crosslinking. Collagen, fibrin, and gelatin are derived from vertebrates and contain cell adhesion signaling markers, while other natural materials, like alginate and agarose, are derived from organisms like plants and algae, so they do not contain those markers. There are a variety of naturally-derived hydrogels that all have their strengths and weaknesses in the EBB realm.

Agarose. Agarose is a polysaccharide molecule that creates a tough hydrogel that undergoes slow gelation at low temperatures.⁵³ The high viscosity of agarose allows it to be very suitable for EBB printing. It provides great stability and allows for the printing of thick scaffolds, but may not be completely suitable for cells to thrive in, as it has low cell spreading and adhesion.⁵⁴ The fact that agarose hydrogels are strong but struggle with cell viability lends them to be used as physical support structures to be used with other hydrogels that can do a better job of supporting cell function. Agarose will liquefy at higher temperatures.⁵⁵ This means that it can be used as a sacrificial material when making scaffolds. By printing it alongside another hydrogel that does not liquefy, the printed agarose can be melted out, leaving open space in the scaffolds. The use of agarose in EBB will take advantage of its physical properties.

Alginate. Alginate is derived from seaweed and algae. It is a shear thinning hydrogel that quickly becomes tough by crosslinking it with calcium ions.⁵⁶ Common calcium ion solutions used to crosslink alginate are calcium chloride (CaCl_2) and calcium sulfate (CaSO_4).⁵⁷ The ease of crosslinking of alginate allows it be done quickly, which makes the alginate hold its printed shape very well (Figure 2.3A). The alginate scaffold can be quickly put into the calcium solution, including by using a coaxial nozzle,⁵⁸ to create a stiff hydrogel that holds its shape very well. Alginate can function in EBB at a range of concentrations. A higher alginate concentration will allow the gel to

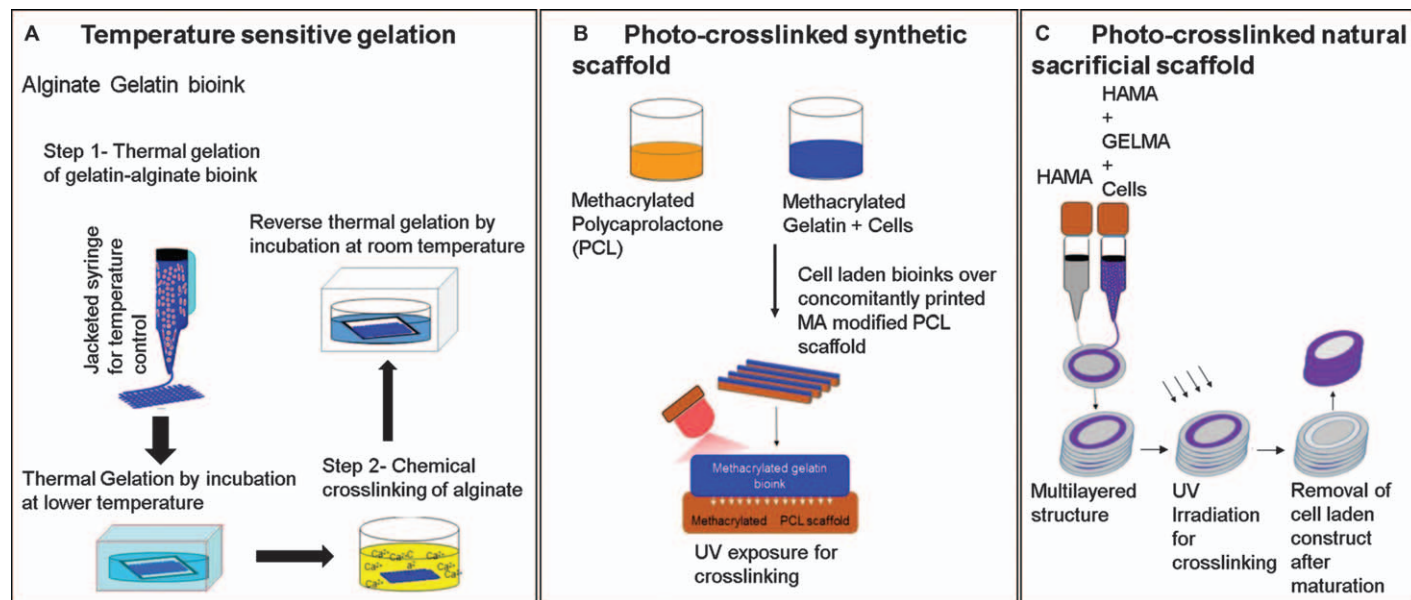


Figure 2.3 Printed bioink crosslinking methods. (A) Thermal gelation and chemical crosslinking process of an alginate gelatin bioink. (B) Photocrosslinking of PCL and Me-Gel scaffold. (C) Photocrosslinking with a sacrificial scaffold. Reproduced from ref. 24, <http://dx.doi.org/10.3390/molecules21060685>, under the terms of the CC BY 4.0 license, <https://creativecommons.org/licenses/by/4.0/>.

be more mechanically stable, while a lower alginate concentration allows for better cell viability, allowing for more effective cell encapsulation. At a low concentration the hydrogels can be too soft.²⁴ Alginate can struggle with cell viability, as it can lack cell binding domains,⁵⁹ but there are solutions. Alginate can be chemically modified to overcome its problems. Chemical modification can be done to increase the cellular functions in alginate hydrogels, as well as to provide a wider range of mechanical properties.⁶⁰ The range of mechanical properties that can be used, as well as its slight ability to support cellular function, makes alginate a good material for EBB. It can be especially helpful as a blend with other materials that complement its properties.

Chitosan. Chitosan is a polysaccharide derived from chitin.⁶¹ Chitosan hydrogels have a high viscosity, which is a good thing for EBB, but they struggle with shape fidelity and can be fragile when crosslinked. This means that they can print well but struggle to hold their shape afterward. Knowing this, chitosan can be added to other hydrogel materials to increase the printability of those gels.³⁰ Chitosan hydrogels are nontoxic, biodegradable, and even have anti-fungal and anti-bacterial properties.⁶² These properties may encourage the use of chitosan for EBB in areas that need high cell viability in an environment that could be a site for potential infection. Chitosan hydrogels undergo ionic crosslinking, commonly using acetic acid in a pH-mediated reaction. This ionic crosslinking can be easily dissociated under physiological conditions, so although chitosan can support cells, they might not last too long in chitosan by itself. Blending chitosan with other hydrogel materials is a good way to take advantage of its positives and to avoid its negatives.

Collagen. Collagen is the most abundant protein in animal ECM.⁶³ There are many types of collagen, but the most commonly used one in EBB is collagen type I.^{31,64} Since collagen is essential in cell ECM, it is very good at supporting cell growth and function, as it enhances cell attachment and growth.⁶⁵ It even does a good job of protecting cells during the extrusion process.²⁴ Collagen can be crosslinked under physiological conditions, like thermal crosslinking.⁶⁶ The physical crosslinking method is easy on the cells, so the cells will not be damaged during the crosslinking process. These factors make collagen a great choice for a hydrogel material requiring cell growth and function support, and it may be helpful when encapsulating cells that are more sensitive to their living environments. One major problem is that hydrogels made of only collagen have low viscosity and slow gelation,⁶⁷ which makes it difficult for use in EBB and causes low shape fidelity. A way to overcome this problem is to mix the collagen with another, more supportive, material for increased mechanical properties. This will allow better printing of the hydrogel, as well as the good support of cellular function and growth by the collagen.

Fibrin. Mixing fibrinogen and thrombin in the presence of calcium creates the fibrin protein.⁶⁸ Fibrin alone is rarely used in EBB due to several factors and may have more downfalls than positive traits. Fibrin has excellent

cellular support properties, but its mechanical properties are poor for EBB. Hydrogels made of fibrin do a great job of supporting cellular function, growth, and proliferation due to fibrin's great cell adherence properties and bioactivity. Fibrin also has good mechanical strength after full crosslinking, but in its pure form, it has very low viscosity.⁶⁹ This makes it extremely difficult to extrude and hold its shape during the printing process. The fibrin crosslinking is even quickly reversible at body temperature and degrades quickly.⁷⁰ This means that it cannot hold its shape or the encapsulated cells when implanted, or even incubated at higher temperatures. There can even be immune reactions to fibrin,² which can be extremely dangerous if it happens if the hydrogels are implanted. If fibrin is going to be used in EBB it should be used as an additive to another material so that it enhances the cell support.^{71,72}

Gelatin. Gelatin is hydrolyzed collagen from the skin of animals and is categorized into type A and type B depending on the source.⁷³ It is a great material to use for EBB, as it combines both mechanical and biological properties into one hydrogel material. Gelatin is great for cellular support. It has low immunogenicity and supports cell differentiation, adhesion, migration, and proliferation.⁷⁴ Using gelatin to encapsulate cells for EBB will allow them to survive long-term culture, grow, and function to a high capacity. Gelatin crosslinks to form a gel at low temperatures, which makes it fairly easy to work with in EBB.^{34,75} The printing of gelatin can be difficult to get perfect because gelatin printing might have a lower resolution than some other gels, low shape fidelity, and low stress shielding during printing. The resolution and fidelity problems can be easily overcome by doing some pre-crosslinking and/or further crosslinking right after the printing is finished. Gelatin is a fairly versatile material. It can be modified to possess enhanced mechanical, biological, or crosslinking properties. Modifying gelatin to methacrylated gelatin (Me-Gel), for example, allows the hydrogel retain some biological properties while increasing the printing resolution.^{76,77} The shape fidelity is also able to be increased by using Me-Gel because it is able to be photo-crosslinked immediately, holding its shape while further crosslinking occurs (Figure 2.3B). Gelatin may be a good hydrogel material for EBB, but modifying it and mixing it with other types of materials can still enhance its abilities.

Hyaluronic Acid. HA is a major ECM component from cartilage and connective tissues⁷⁸ and is used in the maintenance of the structural integrity and homeostasis of tissue, ECM hydration, and cell growth, migration, and differentiation.^{79,80} HA hydrogels have great biocompatibility and have controllable mechanics, architecture, and degradation based on how they are used.^{81,82} HA makes a hydrogel with a high viscosity and stress shielding, which is good for EBB, but it can have low shape fidelity and slow gelation.⁸³ HA can be used as a support material in hydrogel blends to increase the viscosity and printability.⁸⁴ Modifying HA can fix some problems with the mechanical properties of HA hydrogels. Modifying HA can enhance

the rheological properties and allows for other crosslinking methods.⁸⁵ Methacrylated hyaluronic acid (Me-HA) allows for photocrosslinking of the hydrogel,⁸⁶ which will increase shape fidelity, as it can be done instantly. Blending Me-HA and Me-Gel can create a hydrogel that is good for EBB and the encapsulated cell viability and function after printing.³³ This mixture can be used in the printing of a sacrificial material for construction of a cell-laden scaffold (Figure 2.3C). Other blends work well, since HA is great for cells and increases printability.^{84,87}

2.1.3.1.2 Synthetic Hydrogels. Synthetic hydrogels are useful because they can be adapted to meet the needs of the EBB process without being stuck with using what nature gives as the properties. They can be modified with crosslinkable functional groups, as well as domains which increase structural and mechanical properties.¹ Synthetic hydrogels can even be modified to respond to electrical or magnetic stimuli.⁸⁸ A problem with synthetic hydrogels is that they usually do not contain cell binding domains, so they may need to be modified if these domains are desired.⁸⁹ A few main synthetic hydrogels being used as bioinks in EBB are pluronic acid and PEG.⁹⁰

2.1.3.2 Decellularized ECM

ECM is a local environment made up of a set of specific molecules secreted by cells that promotes cell attachment, proliferation, cell signaling, and tissue development.⁹¹ It can be isolated from a variety of animal tissues.^{92,93} Since ECM is the natural cellular environment, it is beneficial to use it as a bioink in EBB to support cell function and growth. To create decellularized ECM (dECM) for use as a bioink, physical, chemical, or enzymatic methods are used to remove the cells, while maintaining the ECM components.^{94,95} The remaining ECM is powdered then dissolved in a cell-friendly buffer, creating a gel-like material that is able to be used in EBB.⁴¹ In general, dECM bioinks have low viscosity, mechanical strength, and shape fidelity, but this can vary with the concentration and location of origin of the dECM.^{24,55} The local mechanical properties may closely mimic the mechanical properties and the microenvironment of the native cells, thus giving high cell viability. Since dECM bioinks have a hard time holding their shape, they need some assistance with shape retention. This can be done by crosslinking the gel further or providing support structures.⁴⁹ Some physical crosslinking can be done by incubating the gel in a heated, humidified chamber.⁴⁹ This can help to hold the shape, but it may not be perfect, so further support structures may be needed. The bioinks made from dECM are fantastic at supporting cell function and viability, but before they can be used for a wide variety of applications using EBB, the fact that the dECM bioinks have low shape fidelity and biodegrade too quickly needs to be overcome.

2.1.3.3 Extrusion-based Hybrid Bioprinting Materials

Sometimes an EBB scaffold needs better mechanical properties than a standard bioink can provide. EBB's abilities allow for the printing of bio-compatible thermoplastic polymers that can provide the support needed. Although they might not technically be able to be considered bioinks because they cannot support cells inside of them during the printing process, there are materials that can be printed using EBB by melting and extruding them. These materials are usually polymers that can melt and harden quickly to form their printed shapes. They may require temperatures to melt that are too high for cells to survive in them during the extrusion process. Although they cannot encapsulate cells, most of these materials are biocompatible and can actually be seeded with cells after printing.⁹⁶ Hybrid bioprinting combines the non-bioink with a bioink by creating a structure with the non-bioink for support and printing the bioink in the gaps, to produce the desired effect.⁹⁷ Some of the materials that can be used in hybrid EBB are polycaprolactone (PCL), polymethyl methacrylate (PMMA), polylactic acid (PLA), some glasses, and others.⁹⁸ A major use of these hard materials is to provide mechanical support and strength for a bioprinted scaffold. Using these types of materials can be beneficial to enhance EBB techniques.

2.1.4 Applications of EBB

EBB has been used in a wide variety of applications, and the number is always growing as more knowledge is gained and new materials are being made and tested. It can be used with or without cells, with hydrogels and plastics, in multi-material systems, and other applications. Common applications for EBB are tissue engineering, tissue models, and drug fabrication.⁹⁹ By harnessing the full capabilities of EBB, along with its compatible materials, many breakthroughs can be made.

2.1.4.1 Tissue Engineering

The main area of use of EBB, and bioprinting in general, is in tissue engineering. Tissue engineering is a promising field and can range from soft tissue, such as muscles and organs, to hard tissue, such as bone and cartilage. The ultimate goal of tissue engineering is to create functional tissues and solid organs that can be implanted, and EBB is able to print complex tissue patterns with multiple bioinks. This can allow the EBB system to bioprint specific cells and materials into the complex structures needed for tissues and organs to function properly. Some tissues and organs that have been printed using EBB are blood vessels,¹⁰⁰ vasculature,³⁵ bone,^{97,101} cartilage,^{102,103} heart valves,^{33,104} cardiac tissue,¹⁰⁵ adipose tissue,¹⁰⁶ liver tissue,¹⁰³ skin,¹⁰⁷ even a bionically integrated ear,¹⁰⁸ and basically any other tissue. A process of using multi-material hybrid bioprinting with imaging can be used to produce a craniofacial bone construct (Figure 2.4). 3D

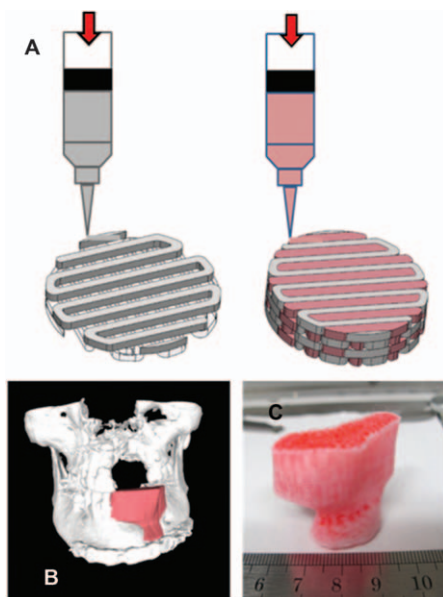


Figure 2.4 (A) Multi-material scaffold printing model. (B) CT scan of a craniofacial defect with filled defect. (C) Multi-material print of craniofacial defect scaffold. (B) and (C) reprinted from ref. 44 with permission from The Royal Society of Chemistry.

printing can be used to create a number of tissue engineering constructs, as well as drug delivery constructs (Figure 2.5). Tissue engineering is a way off of producing completely functional organs from these tissues, but as EBB technology and experience advance, the ability of EBB systems to print the complex tissue systems needed for complex organs to function properly will increase. Some hurdles in EBB of tissues and organs are perfectly organizing the complex tissue structures that are needed, mimicking the natural micro- and macro-environments of the cells and tissues, incorporating vasculature into the tissue, and more.

2.1.4.2 Tissue Models

Tissue models are made so that the testing of things like drugs, toxicity, and diseases can be done without having to use the actual tissue of interest. Allowing for *in vitro* testing makes for an easier and cheaper way to test the tissue reaction than using the real tissue every time, especially *in vivo*. Cells mimic *in vivo* behavior more closely when they are in 3D conditions, rather than in 2D culture.¹⁰⁹ The 3D conditions for tissue modeling are easier to make than the implantable tissue because it does not need to be fully functional. It just needs to correctly represent the behavior of the cells and tissues under the correct conditions. If their reactions are similar to a drug, material,

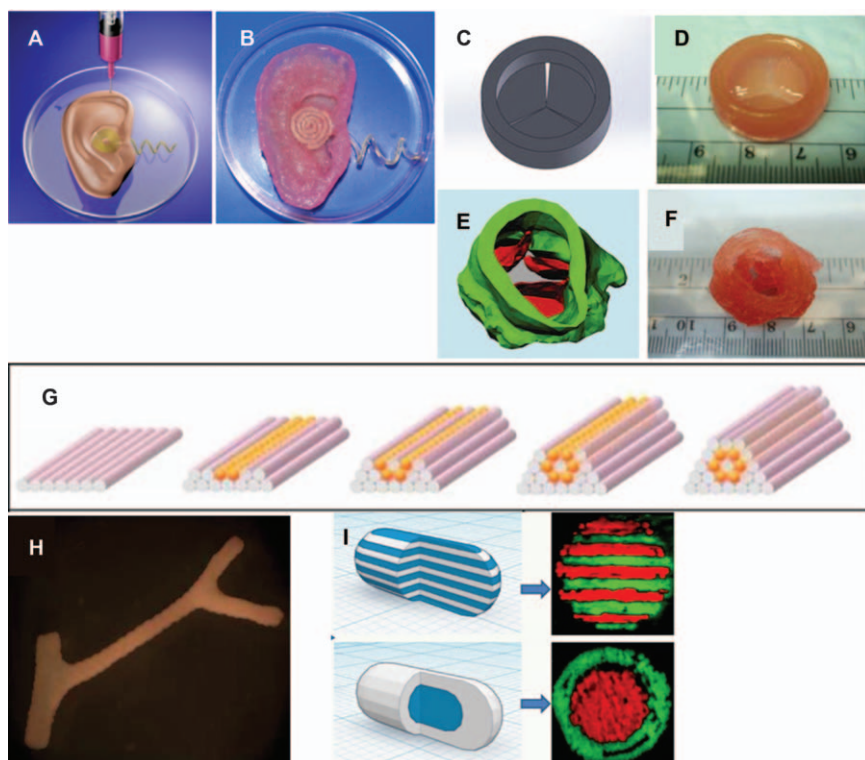


Figure 2.5 Applications of EBB. (A–B) 3D printed bioinoc ear. (A) Model of bionic ear. (B) EBB printed bionic ear. (C–D) 3D printed heart trileaflet valve. (C) Model of trileaflet valve. (D) EBB 3D printed trileaflet valve. (E–F) 3D printed heart aortic valve. (E) 3D model of an aortic heart valve. (F) EBB printed aortic heart valve. (G) Process of 3D printing a blood vessel using a sacrificial scaffold material. (H) Printed blood vessel using a sacrificial scaffold. (I) 3D printed drugs into specified patterns with varying material layers and one material encapsulated in another. (A) and (B) reprinted with permission from ref. 108, Copyright 2013 American Chemical Society. (C) and (D) reprinted from ref. 33 with permission from Elsevier Ltd., Copyright 2013 Acta Materialia Inc. (E) and (F) reprinted with permission from ref. 34, Copyright 2012 Wiley Periodicals, Inc. (G) and (H) reprinted from ref. 100 with permission from Elsevier Ltd. I reproduced from ref. 113 with permission from American Chemical Society, Copyright 2015.

or disease, then the tissue model has done its job. Tissue models can even be created so that different functional parts of a tissue can be studied separately under the test conditions. The tissue models can mimic hard tissue like bone and cartilage, as well as soft tissue like organs and vasculature.¹⁰⁹ They are even able to mimic cancer in some models.^{110,111} EBB is great for creating tissue models because it can easily model cells into defined 3D shapes and positions. It can create complex patterns of cells, so that growth and reaction of the cells can be dictated to be as close to the native tissue as possible.

2.1.4.3 Drug Fabrication

EBB is good for drug fabrication because it can print a variety of materials in a complex pattern, while maintaining a low printing temperature. The EBB process allows for the use of multiple types of drug materials. It has a low stress effect during the printing process, so it can print conventional drugs and materials like DNA plasmids without degrading them.¹¹² This allows for EBB use in a broad range of drug and therapy applications.

Since EBB can precisely print materials into specified patterns, complex structures can be made in the drug delivery systems. By adjusting the materials used and the printing pattern, the drug delivery system can be adjusted for the desired controlled release. Some complex printed structures that can add to the controllability of EBB of drugs are layer-by-layer deposition of different materials or drugs, a core surrounded by a different material, porous constructs and even varying porosities, and others.¹¹³

Another perk of using EBB in drug fabrication is the ability to personalize the drug to the person who needs it.¹¹⁴ Since it is 3D printed, it could also be made on demand. Different sizes and doses can be easily made based on the user's body weight, age, and other parameters.⁹⁹ This could be an easy way to make sure everyone gets the personalized and correct treatment.

The main advantages of EBB in drug fabrication are its flexibility in materials and drugs that can be used, speed, personalization, precision with complex structures, and the ability to print multiple materials at once. EBB does have downfalls though. Some materials that can be used in EBB drug fabrication can contain organic solvents and may have residual solvent remaining after printing, and there can be shrinking or deformation post printing.

2.1.5 Future Directions

The future of EBB seems to be a bright one. It has applications in tissue engineering, tissue modeling, drug fabrication, and more. As technology advances and the machines become more precise and have increased capabilities, even more applications may come into play for EBB. More material options that work in EBB can be a basis on which to advance the capabilities of EBB and bioprinting in general. There needs to be an optimization of bioinks for specific applications, as well as the creation of new bioinks and bioink combinations that can nearly mimic tissue.

There is a real possibility of printed organs and tissues becoming functional and implantable. It will take the step up from proving the functioning of cells and tissues in small bioink structures to larger-scale tissues. The seemingly biggest hurdle for this right now is the integration of vasculature into the printed constructs, whether it be natural or artificial. The vasculature is important for getting nutrients to the cells and tissue throughout its growth and functioning stages, and EBB can have the ability to print this vasculature. A big step in tissue engineering is to create clinically functional

tissues and organs, and EBB is a technology that will help to achieve this goal. This might take combining multiple cell printing and growth techniques into one process, and EBB is a promising technique to incorporate into those processes.

The future of drug delivery in EBB can be targeted drug delivery, controlled release, and gene therapies. Targeted and controlled delivery can be advanced using EBB by using materials with varying properties and loaded with drugs in specifically defined patterns. This will allow the release of the drugs at certain times and locations in the body. Advances in bioinks, drugs, targeting methods, and printed patterns will allow for EBB to reach its full potential in targeted drug delivery and controlled release. Since the EBB process is fairly gentle on the printed materials, it can be used for gene therapy. It would allow the printing of drugs and the small particles that the genes are loaded in without degrading them. Using the degradation patterns that EBB allows, with the gene therapy techniques, higher efficiency and effectiveness of the gene therapy drugs can be achieved.

EBB has a bright future for use in multiple techniques. Technological advances in EBB itself, materials, and other technologies will allow EBB to progress to its full potential. There may even be new platforms for EBB to be used in that have not even been thought of yet.

2.1.6 Conclusion

EBB is a fairly simple and effective system for creating spatially defined 3D constructs containing living cells or biological factors. It has flexibility in its uses, depending on the cells and bioinks used in the printing process. EBB has applications in tissue engineering, tissue models, drug fabrication, and others. The future of EBB also looks to be a promising one. Technological advances may make EBB an increasingly viable method of producing quality clinical products in an efficient and effective manner.

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CHAPTER 3

Microfluidic Platforms for Biofabrication and 3D Tissue Modeling

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3.1 Introduction

The function of mammalian tissue relies greatly on the microscale tissue architecture into which specific types of cells are three-dimensionally arranged. Three-dimensional (3D) modeling of tissues with these microscale tissue architectures can provide great insights into the governing mechanisms for tissue formation, function, and failure; such tissue models will be used to solve problems in the field of tissue engineering and regenerative medicine. To replicate these microscale tissue architectures and observe cell behaviors inside these architectures, technologies for handling, observing and stimulating the cells with microscale resolution are required. Microfluidic technology—the technology that deals with the behavior, precise control and manipulation of fluids that are geometrically constrained to a small scale—is one of the most promising candidate technologies for the fabrication and modeling of 3D tissues with microscale architectures.

In this book chapter, we will first briefly introduce the fundamental concepts in microfluidics, followed by the common practices to fabricate

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microfluidic chips (devices). Then, we will review the application of microfluidics as platforms for biofabrication and 3D tissue modeling. The state-of-the-art microfluidic platforms can be categorized into two major types: tissue-off-chip (*fab-only*) platforms, and tissue-on-chip (*fabless* and *more-than-fab*) platforms (Figure 3.1). The tissue-off-chip platforms focus on the fabrication of microtissues using the microfluidic chips; after fabrication, the fabricated tissues are taken off the chips, thus these platforms can be also referred to as *fab-only*. The tissue-on-chip platforms focus on the construction/installation, sensing/stimulation of the tissues on microfluidic chips. The tissue-on-chip platforms can be sub-categorized into two different types: *fabless* and *more-than-fab*. The *fabless* type, as its name would suggest, performs on-chip housing/installation, sensing/stimulation of tissues which are either isolated from animal body or pre-fabricated using the tissue-off-chip platforms. The *more-than-fab* type, can not only construct tissues on-chip, but also perform on-chip manipulation, sensing/stimulation of the tissues.

3.2 A Brief Overview of Microfluidics

Microfluidics is the science and technology of systems that process or manipulate small (10^{-9} to 10^{-18} litres) amounts of fluids, using channels with dimensions of tens to hundreds of micrometres.¹ The experiments of microfluidics are often performed on microfluidic “chips”, which contain patterned channels/reservoirs of micrometre-level size.

One of the simplest examples of the microfluidic fabrication process might be the photolithography process which is used to fabricate the micro reservoir array (Figure 3.2(a)). The most prevalent material is polydimethylsiloxane (PDMS), an inexpensive, non-toxic, optically transparent and mechanically robust elastomer; using PDMS, one can either fabricate patterns of proteins following the process described in Figure 3.2(b), or fabricate converging channels on top of slide glass following the process described in Figure 3.2(c); the relative processes are also called “soft lithography.”²

It is worthwhile mentioning that, for microfluidics, the flow in these chips are typically in laminar flow regime; under such regime, fluids behave more orderly comparing to macroscale flows (usually chaotic and easy to generate vortices). As a demonstration of the laminar flow, the two fluids (dyed using different colors) in the converging channels form clearly defined the boundary in between each other (Figure 3.2(c)).³ For further reading of the interested readers, there are many nicely written textbooks covering microfluidics with concentrations on its physics,^{4,5} fabrication⁶ and biological applications.⁷

3.3 Tissue-off-chip (*fab-only*) Platforms for Biofabrication

In this section, we focus on the tissue-off-chip platforms that perform the fabrication of microtissues using microfluidic chips; after fabrication,

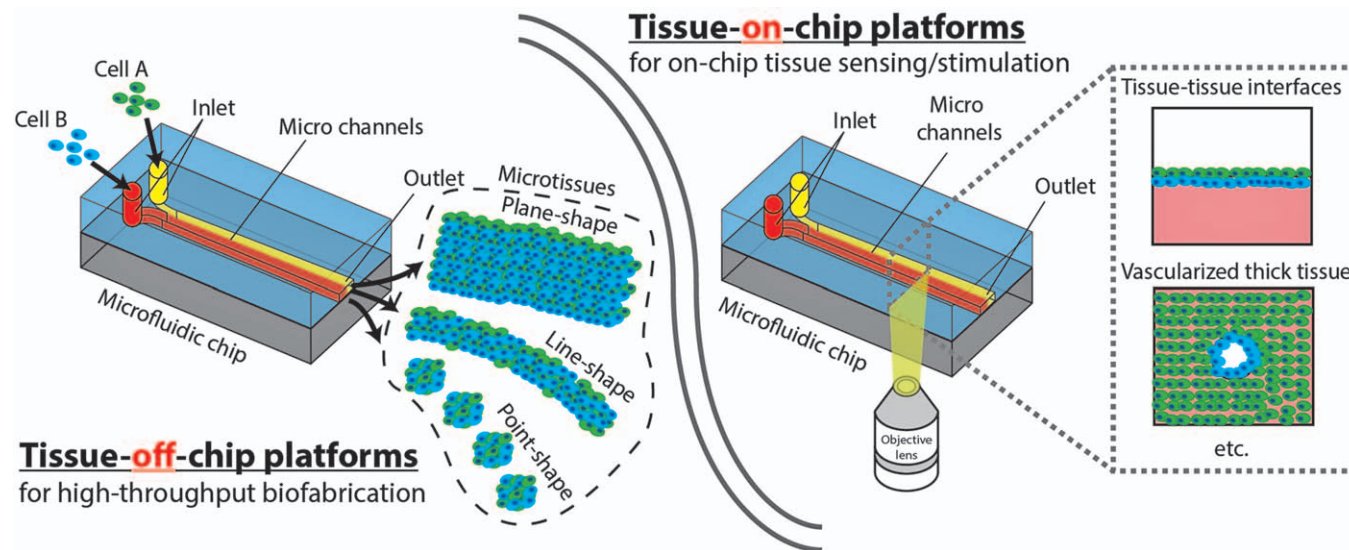


Figure 3.1 Tissue-off-chip and tissue-on-chip platforms. The tissue-off-chip platforms focus on the high-throughput fabrication of microtissues using the microfluidic chips; after fabrication, the fabricated tissues are taken off the chips. The tissue-on-chip platforms focus on the construction and installation, and sensing and stimulation of the tissues on microfluidic chips; modeling of tissues can be performed based on the tissue behaviors and responses in accordance to specific environmental stimulations.

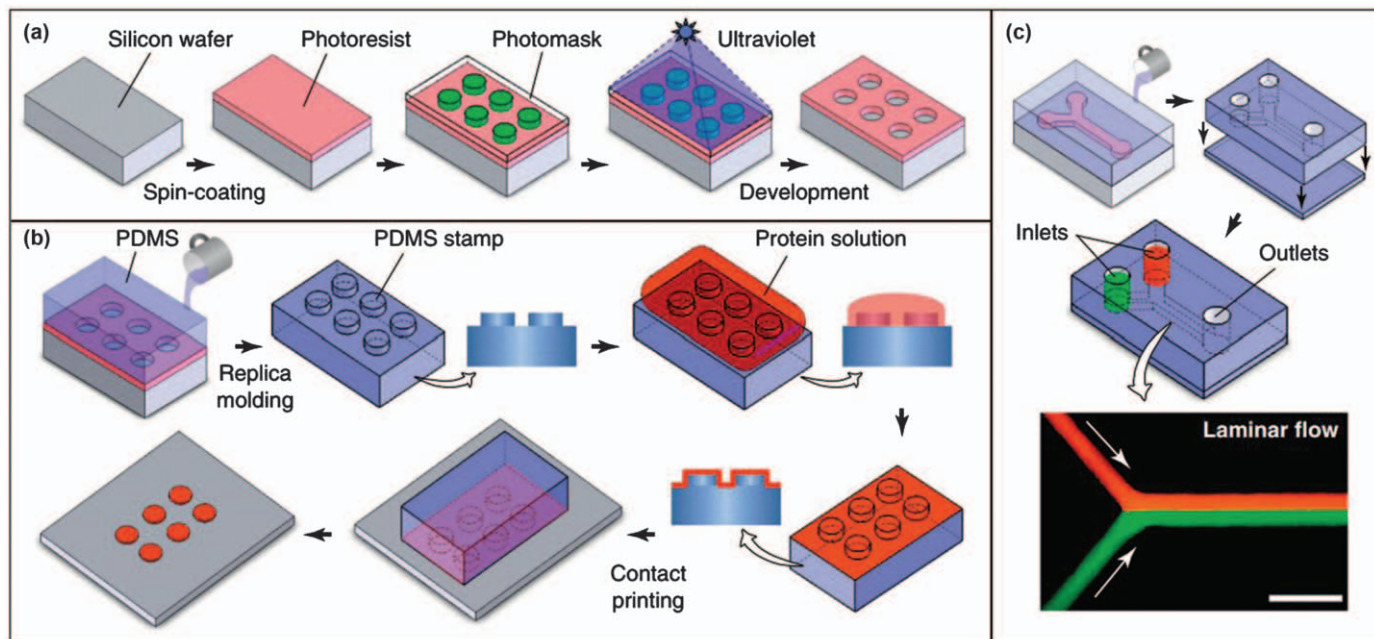


Figure 3.2 Microfluidic fabrication techniques for biofabrication and 3D tissue modeling. (a) Photolithography techniques for the fabrication of micro reservoirs array. (b) Patterning of proteins using "soft lithography" techniques. (c) Microfluidic fabrication of converging channels. Reproduced from ref. 3 with permission from Elsevier, Copyright 2011.

the fabricated tissues are taken off the chips for further culture and investigations, thus these platforms can be also referred to as *fab-only*. One of the important features of the *fab-only* platforms is the reusability of the microfluidic chips; since the fabricated tissues are taken off the chips after fabrication, the chips can be immediately reused after a brief clean up. Another advantageous feature of the *fab-only* platform is the high fabrication throughput; point-shaped, line-shaped and plane-shaped microtissues can be fabricated *en masse* in a non-stop manner and the fabrication can be further parallelized by creating arrays.⁸

Here, we summarize three typical shapes of microtissues which can be fabricated using the microfluidic techniques: point-shape, line-shape, and plane-shape (Figure 3.3).

3.3.1 Microfluidic Fabrication of Point-shaped Microtissues

Point-shaped microtissues are the ones in sphere shape, polyhedron shape, and shapes with a small difference between the lengths of the major and minor axes.

As a major type of 3D cell culture, numerous spheroid fabrication techniques have been proposed, such as hanging-drop culture,⁹ microfluidic chips,^{10,11} and cultures on a low-adhesive substrate or wells.¹² Amongst the microfluidic chips methods, hanging-drop culture with chemical gradient¹⁰ (Figure 3.4(a)) and microwell-based spheroid culture⁹ (Figure 3.4(b)) are the most representative ones. Microfluidic-based methods allow continuous perfusion for nutrient supply and waste disposal, precise dose of drugs and convenient liquid sampling for pharmaceutical tests. However, for the formation of cellular spheroids, microfluidics is not the only technical option, the role of microfluidics in these proposed platforms^{10,11} is more assistive; microfluidics contribute significantly to the perfusion and sampling processes rather than the spheroid formation process. To take more advantages of microfluidic technology, microfluidic chips are used for the formation of cell-laden hydrogel beads; empowered by microfluidic techniques, fabrication of cell-laden hydrogel beads with high throughput, high geometrical definition and uniformity can be achieved.

The formation of cell-laden hydrogel beads is based on the microfluidic generation of liquid droplets. T-junction microchannel chips^{13–16} and 2-D microfluidic flow-focusing chips^{17–19} are the representative microfluidic chips for droplet generation. Figure 3.4(c) shows the fabrication of cell-laden alginate beads using a T-junction microfluidic chip.²⁰ The hydrogel precursor was chosen as sodium alginate (containing CaCO₃) and was formed into microbeads using oil (with surfactants and acetic acid) as the continuous phase of water/oil emulsion. Gentle gelation of alginate was triggered upon contact of sodium alginate with oil. Figure 3.4(d) shows the fabrication of cell-laden synthetic polymer beads using a 2-D flow-focusing

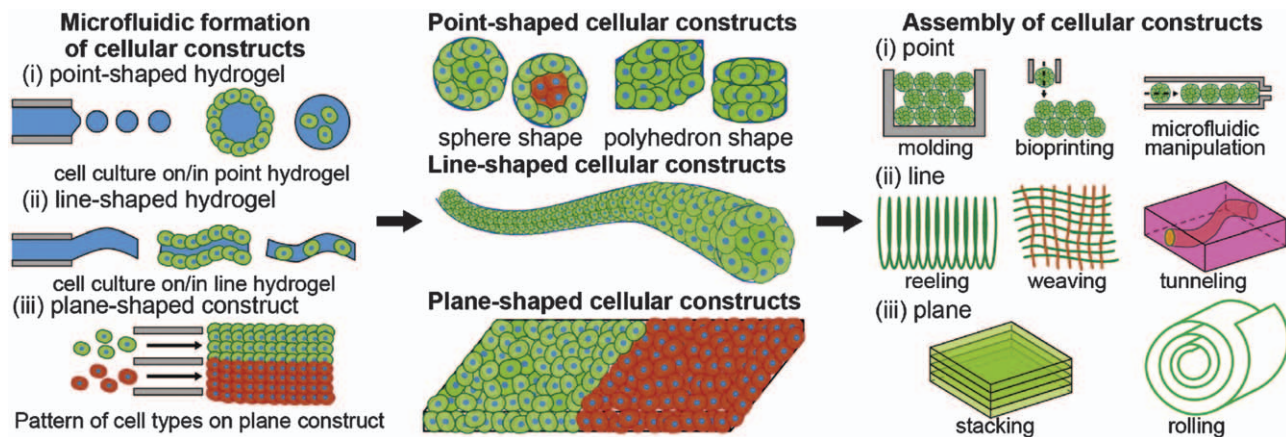


Figure 3.3 Microtissues with various shapes fabricated by microfluidic techniques. Reproduced from ref. 8 with permission from Elsevier, Copyright 2015.

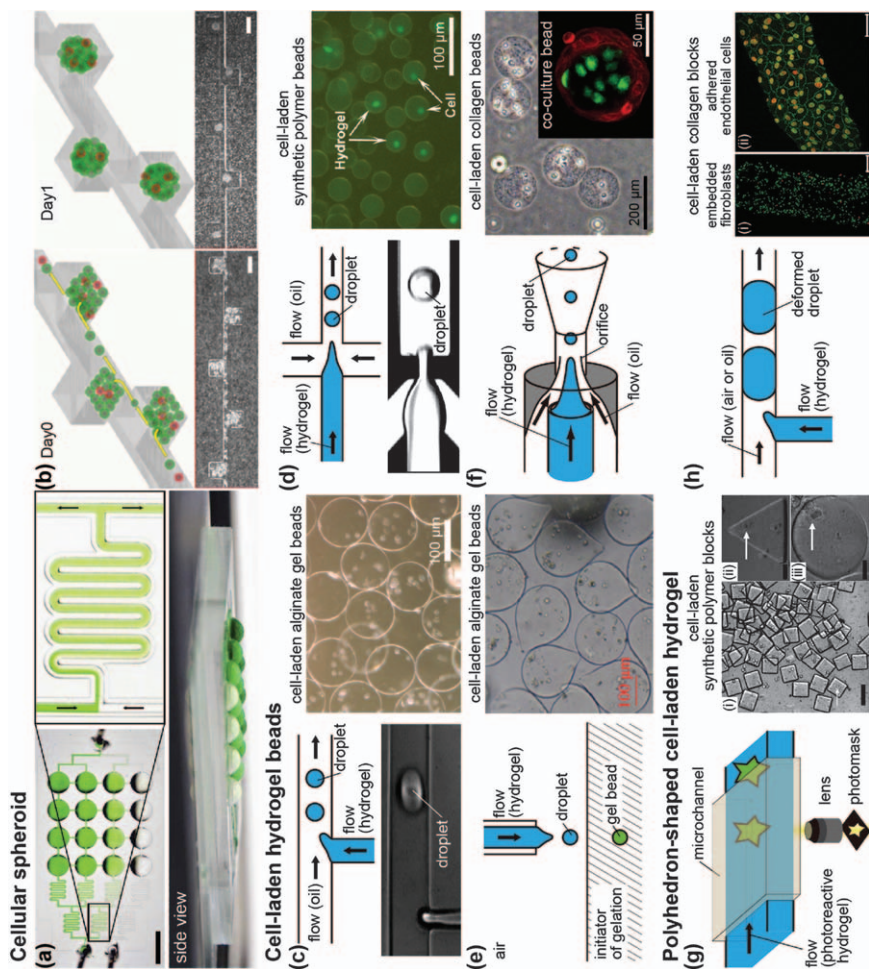
chip.²¹ Huang *et al.* reported a microfluidic device combined with injection needles to generate micro droplets (Figure 3.4(e)); the infusing of the fluids, as well as the spotting as retrieval of the needles into oils, are controlled simultaneously using cleverly designed pneumatic micro-vibrators.²² Recently, with the advancement of high-resolution 3D printing technology, microfluidic chips with co-axial circular channels are proposed and validated for the generation of emulsion droplets²³ and cell-laden hydrogel beads with guaranteed monodispersity and cell viability²⁴ (Figure 3.4(f)).

The formation of polyhedron-shaped cell-laden construct relies on optofluidic²⁵ or rail-assisted approaches.²⁶ The optofluidic approach^{25,27,28} fabricates cell-laden hydrogel blocks by exposing dynamically controllable UV light patterns to photoreactive hydrogel precursors in a flat and transparent microfluidic channel²⁵ (Figure 3.4(g)). The optofluidic approach allows the fabrication of user-defined shapes with high efficiency; after fabrication, the fabricated particles with various shapes can be selectively assembled into neatly aligned patterns.²⁹ The rail-assisted approach is based on the T-junction geometry for droplet generation, however the difference is the utilization of the outlet channel as a “rail” to limit the axial diameter of the giant droplets to form capsule-like rod-shaped constructs²⁶ (Figure 3.4(h)).

3.3.2 Microfluidic Fabrication of Line-shaped Microtissues

Line-shaped microtissues (a.k.a. cell-laden microfibers) are long, thin cellular constructs consisting of cells and hydrogels. The hydrogels could be artificial ECMs, ECMs derived from native tissues or ECMs secreted *in situ* by the cells in the microtissues. Liquid thread formation of the cell-laden materials is the key for the fabrication of the line-shaped microtissues; such thread formation can be achieved using syringe-extrusion,^{30,31} laminar flow flow-focusing microfluidic chips,^{32–38} multi-interfacial polyelectrolyte complexation (MIPC)^{39–41} and electrospinning^{42–44} (Figure 3.5(a)). Depending on the location of the cells, the cell-laden microfibers could be further categorized as surface-type and encapsulation type (Figure 3.5(b)). At the application side, the line-shaped microtissues could be further assembled into hierarchical shapes, or be used as templates for the creation of tunnels; the tissues could be also used as animal-alternative tissue models, and be used as implantable tissue constructs (Figure 3.5(c)).⁴⁵

Microfluidic flow-focusing devices are crucial for line-shaped microtissues fabrication. Yamada *et al.* proposed PDMS-based microfluidic chips with multiple layers of converging junctions (one T-shapes with two more V-shaped junctions); using the microfluidic chip, cell-laden microfibers of 3T3 cells co-cultured with hepatocytes was fabricated to mimic the stromal/parenchymal interaction in native liver tissues⁴⁶ (Figure 3.5(d–e)). Kang *et al.* adds to the complexity of the microfluidic chips by adding *in situ* fabricated pneumatic valves; using this type of microfluidic chips, fabrication of



microfibers with serial/parallel coding was achieved.³⁷ In addition, the microfluidic chips with grooves along the longitudinal axes of the channels was also proposed to fabricate grooved hydrogel microfibers; such fibers could be further seeded with neural cells and the polarity of the cells was found out to be guided by the direction of the grooves.⁴⁷ Shin *et al.* proposed a microfluidic chip based on PDMS substrate with inserted glass capillaries; pulled and tapered glass capillaries with tip inner/outer diameters of 35/60 μm were inserted into another glass capillary to form a flow-focusing geometry, and using the chip cell-laden microfibers was successfully fabricated (Figure 3.5(f–g)).⁴⁸ Onoe *et al.* replaced the soft PDMS substrate with rigid stereolithography-based fluid connectors and constructed sophisticated channel configurations with double co-axial flow-focusing geometries; using such a chip, core-shell type microfibers were fabricated with a cell-laden core composed of soft native ECMs (such as low concentration collagen,³⁴ fibrin,^{35,49} *etc.*). Using the glass-capillary-based microfluidic chips, not only straight microfibers, but also helix/coil-shaped microfibers could be fabricated by tuning the viscosity and flow rate setups to trigger the so-called liquid rope-coil effect in which a thin stream of liquid thread buckles in a periodic fashion (Figure 3.5(h–j)).^{50–52}

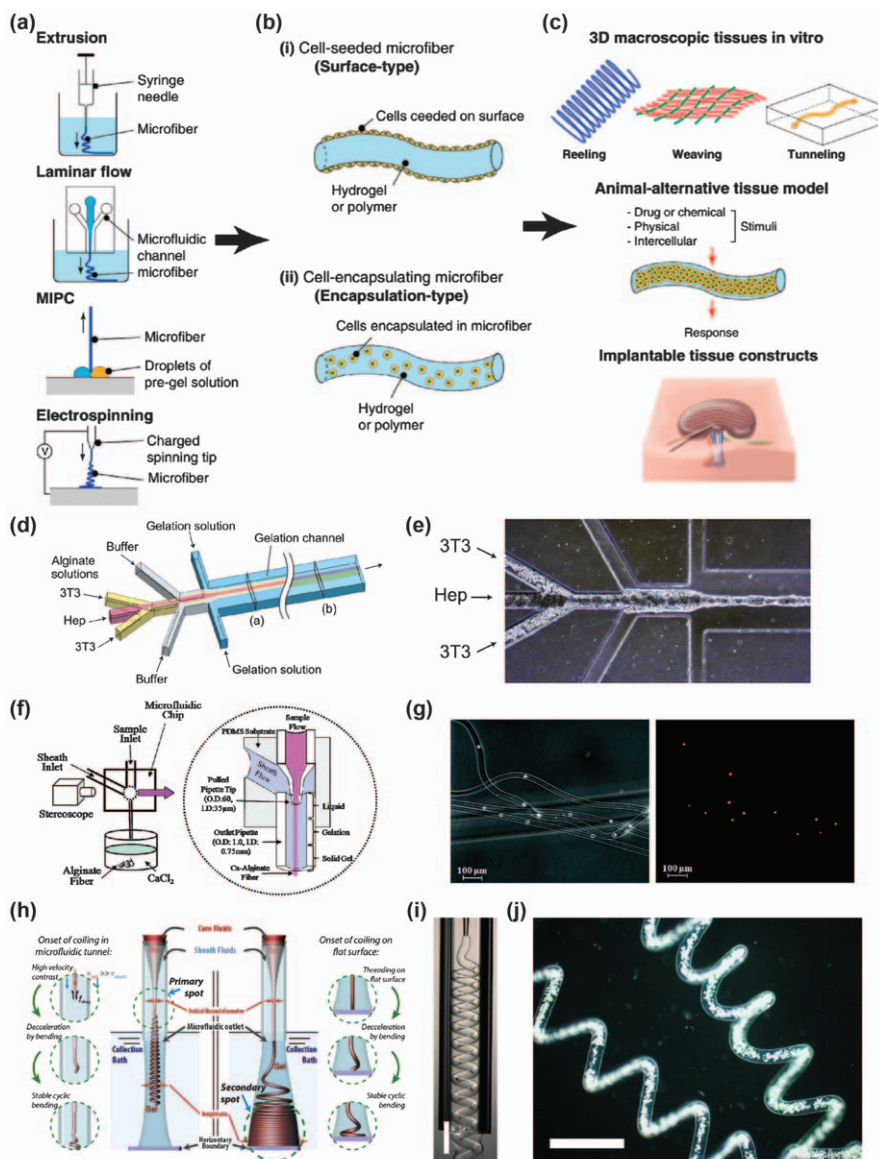
3.3.3 Microfluidic Fabrication of Plane-shaped Microtissues

Plane-shaped microtissues can also be fabricated using microfluidic platforms. During the fabrication process, single/multiple types of cell-laden hydrogel precursors are introduced into wide, flat microchannels, and plane-shaped microtissues can be formed with the crosslinking of hydrogel precursors. The microfluidic channels can facilitate variable arrangement of different cells on the substrates of the microfluidic chips^{53,54} or in the hydrogel sheets⁵⁵ by controlling the composition or gradient of the cell-laden hydrogel precursors.

Figure 3.4 Point-shaped microtissues fabricated using microfluidic platforms. (a) Hanging-drop culture with microfluidic generated chemical gradients.¹⁰ (b) Microwell-based spheroid culture with microfluidic perfusion control.⁹ (c) The fabrication of cell-laden alginate beads using a T-junction microfluidic chip.²⁰ (d) The fabrication of cell-laden synthetic polymer beads using a 2-D flow-focusing chip.²¹ (e) Microfluidic generation of micro droplets and alginate microbeads for cell encapsulation using pneumatic micro-vibrators.²² (f) Generation of cell-laden hydrogel beads with guaranteed monodispersity and cell viability using 3D printed microfluidic flow-focusing chips.²⁴ (g) The optofluidic fabrication of a cell-laden hydrogel block by exposing dynamically controllable UV light patterns to photoreactive hydrogel precursors in a flat and transparent microfluidic channel.²⁵ (h) The rail-assisted approach utilizes T-junction to generate micro droplets and utilize the outlet channel as a “rail” to limit the long axis diameter of the giant droplets to form capsule-like rod-shaped constructs.²⁶ Reproduced from ref. 8 with permission from Elsevier, Copyright 2015.

3.4 Tissue-on-chip (*fabless/more-than-fab*) Platforms for 3D Tissue Modeling

In this section, we focus on the tissue-on-chip platforms that perform the construction/installation, sensing/stimulation of the tissues on microfluidic chips. The tissue-on-chip platforms can be sub-categorized into two different types: *fabless* and *more-than-fab*. The *fabless* type, as its name would suggest, performs on-chip manipulation, sensing/stimulation of tissues which are

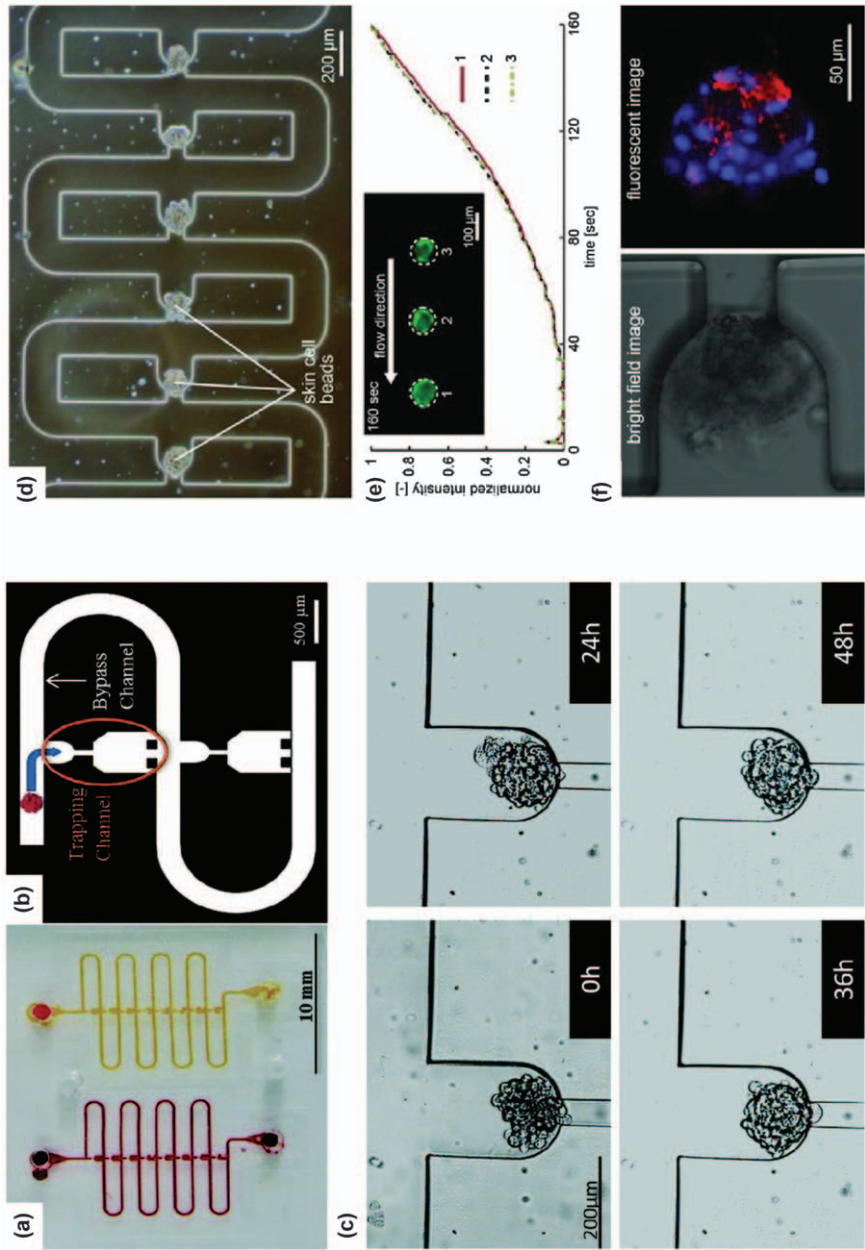


either isolated from an animal body or pre-fabricated using the tissue-off-chip platforms. The *more-than-fab* type, can not only construct tissues on-chip, but also perform on-chip construction, sensing/stimulation of the tissues.

3.4.1 On-chip Tissue Construction and Installation Techniques

The modeling of 3D tissue is based on the static measurement of the tissue composition and morphology, as well as the dynamic recording of tissue behaviors or responses in accordance to specific environmental stimulations. To perform such measurements and recordings on the microfluidic chips, the source of tissues as well as the installation of the source tissues onto the chip are the first fundamental requirements. The source of tissues for microfluidic chips can be categorized into the following three types: biofabricated microtissues, tissues isolated from animals, as well as tissues constructed *in situ* on the microfluidic chips. Biofabricated microtissues are the microtissues fabricated on the tissue-off-chip (*fab-only*) microfluidic platforms, which we have introduced in detail through the previous sections. The tissues isolated from an animal could be directly installed onto the microfluidic chips (in a *fabless* fashion) to perform further

Figure 3.5 Line-shaped microtissues fabricated using microfluidic platforms. (a) Liquid thread formation of the cell-laden materials is the key for the fabrication of the line-shaped microtissues; such thread formation can be achieved using syringe-extrusion, laminar flow flow-focusing microfluidic chips, multi-interfacial polyelectrolyte complexation (MIPC) and electrospinning. (b) Depending on the location of the cells, the cell-laden microfibers could be further categorized as surface-type and encapsulation type: the surface-type (i) cell-laden microfibers have cells on the surface of the microfibers; the encapsulated-type (ii) cell-laden microfibers have cells inside the microfibers. (c) The line-shaped microtissues could be further assembled into hierarchical shapes by reeling, weaving and other fabric manipulation methods, or be used as templates for the creation of tunnels; the tissues could be also used as animal-alternative tissue model, and furtherly be used as implantable tissue constructs. (d–e) PDMS-based microfluidic chips with multiple layers of converging junctions (one T-shapes with two more V-shaped junctions) and the microscopic photo showing the fabrication of the microfibers.⁴⁶ (f–g) 3D flow-focusing channels fabricated based on PDMS substrate with inserted glass capillaries to form the flow-focusing geometry and the cell-laden microfibers generated using the chip.⁴⁸ (h–j) Helix/coil-shaped cell-laden microfibers fabricated by tuning the viscosity and flow rate setups to trigger the so-called liquid rope-coil effect in which a thin stream of liquid thread buckles in periodic fashion.⁵¹ (a)–(c) reproduced from ref. 45 with permission from Elsevier, Copyright 2014. (d) and (e) reproduced from ref. 46 with permission from Elsevier, Copyright 2012. (f) and (g) reproduced from ref. 48 with permission from American Chemical Society, Copyright 2007. (h) and (i) reproduced from ref. 51 with permission from Elsevier, Copyright 2017. (j) courtesy of Minghao Nie, University of Tokyo.

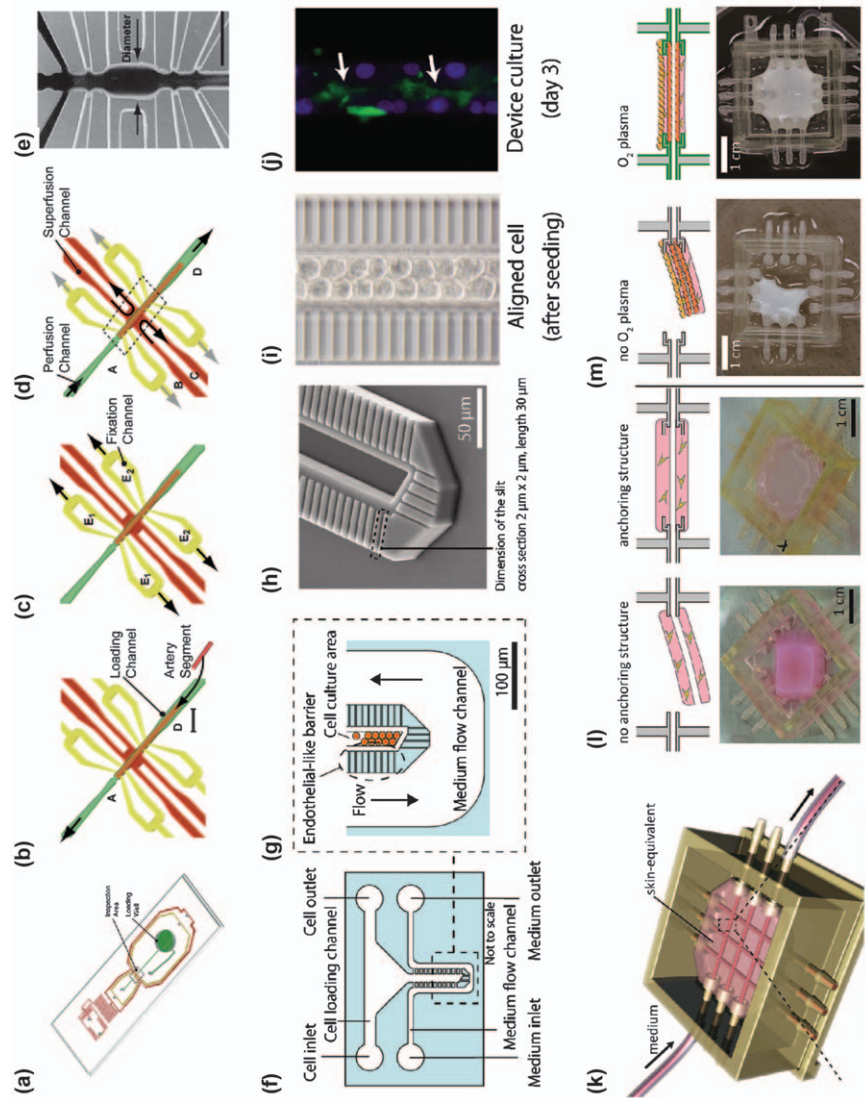


tissue modeling. The tissues could also be constructed *in situ* on the microfluidic chips (in a *more-than-fab* fashion) by infusing cell suspensions into the chips and waiting for the cells to settle and adhere on the surface of the channel inner sides, or to form into cell spheroids in the micro reservoirs of the chips. In the following texts, we will focus on three types of representative techniques for the installation or construction of tissues on-chip: dynamic microarrays for tissue trapping, on-chip tissue housing and/or anchoring techniques and on-chip construction of tissue barriers.

3.4.1.1 Dynamic Microarrays for Tissue Trapping

Microfluidic chips are able to perform the manipulation of micro or sub-macro sized objects; such tasks are generally impossible for traditional manipulation techniques in biology labs to perform. There are many kinds of microfluidic manipulation techniques based on guiding or trapping forces generated using geometrical,⁵⁶ hydrodynamic,⁵⁷ optical-induced thermodynamic,⁵⁷ inertial,⁵⁸ optical,⁵⁹ electrical⁶⁰ or ultrasonic^{61–63} approaches. However, some of these methods are restricted to manipulating small objects on the size of several micrometers or even smaller, thus are only suitable for the manipulation of single cells or even smaller cellular compositions (DNA, RNA, *etc.*). For the manipulation of microtissues on the size of several hundred microns or even larger, hydrodynamic force-based methods are the most popular due to their ease in generating high enough magnitudes of forces, ease of chip fabrication (no cumbersome electrode fabrication is needed), design flexibility (functions depend mostly on channel geometries) and ease of fluidic input control (syringe pump-based). Tan *et al.* proposed a microchannel design with basic units composing of square wave-shaped loop channels superimposed onto a straight channel, with narrowed regions along the straight channel functioning as traps; using this design, transportation, immobilization, infusion of reagents, observation, and retrieval of spherical shaped micro sized objects in a single integrated device was successfully demonstrated.⁵⁷ Inspired by the micro-channel design, Ruppen *et al.* performed high-throughput chemiresistive testing of multicellular pleural cancer spheroids (Figure 3.6(a–c));⁶⁴ Das *et al.*

Figure 3.6 Dynamic microarrays-based tissue trapping and assay. (a–c) Chip design, trapping unit schematic and demonstration of tissue monitoring up to 48 h under perfusion condition.⁶⁴ (d) Phase-contrast microscopic image of skin microbeads trapped using dynamic microarrays; (e) fluorescent image and brightness change of the skin cell beads labeled using the Live (green) and Dead (red) assay; (f) bright-field and fluorescent images of a trapped skin cell bead after 48 h culture in the dynamic microarray device (visualized type-VII collagen (red) and cell nuclei (blue) by immunostain).²⁴ (a)–(c) reproduced from ref. 64 with permission of The Royal Society of Chemistry. (d)–(f) reproduced from ref. 24 with permission from John Wiley and Sons, Copyright 2013 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.



achieved the spheroid-based modeling of ovarian cancer with empirical chemosensitivity testing.⁶⁵ Morimoto *et al.* also demonstrated the highly efficient handling of skin cell beads using similar microchannel designs (Figure 3.6(d–f)).²⁴

3.4.1.2 On-chip Tissue Housing and Anchoring Techniques

On-chip housing and anchoring of tissues deals with tissues isolated from an animal body or tissues formed *in situ* on the microfluidic chips.

On-chip housing techniques are used to fix tissues isolated from an animal body or tissues formed *in situ* onto the microfluidic chips. Compared to artificially reconstructed tissues which usually lack some of the features of native tissues, native tissue isolated from an animal body can provide more information on the target tissues. To perform tissue analysis on microfluidic chips, specially designed channels for the housing as well as stimulation of the tissues are of essential needs. The design of the housing channels varies depending on the different physical properties and morphologies of the target tissues. As a representative work, Guenther *et al.* introduced a fixation channel design to apply suction for the fixation of small arteries; besides the fixation channel design, the proposed microfluidic chip is also able to perform perfusion of the housed artery through its lumen (Figure 3.7(a–e)).⁶⁶ Using the microfluidic chips, constriction of an artery segment in either spatially homogeneous or heterogeneous microenvironments was successfully investigated. Based on this design, Yasotharan *et al.* reported a compact microfluidic platform for the automated, multimodal assessment of intact small blood vessels; immunohistochemical endpoint protein analysis as well as a simultaneous assessment of the time-dependent, agonist-induced calcium and diameter changes of pressurized resistance arteries were performed in a highly automatic manner.⁶⁷ Another example of the housing design is proposed by Nakao *et al.* who fabricated wall structures with perforated side walls to mimic endothelial-like barrier (Figure 3.7(f–h)).⁵⁶ During usage, cells are introduced to the “cell culture area” which is housed

Figure 3.7 On-chip tissue construction and installation techniques. (a–d) Chip schematic and operation principle of the artery-on-a-chip platform and (e) resistance arteries isolated by microdissection and loaded on the microfluidic chip.⁶⁶ (f–g) Schematic of the tissue housing platform with endothelial-like barrier for mimicking the structure of a hepatic cord; (h) SEM image of the fabricated endothelial-like barrier; (i) aligned cells were found in two lines like a hepatic cord; (j) hepatocytes formed bile canaliculi along the hepatic cord-like structure (visualized using CDFDA⁵⁶ assay). (k) Perfusable skin-on-a-chip concept and (l, m) the demonstration of the effectiveness of anchoring design and plasma treatment on the enhancement of tissue fixation on chip.⁷¹ (a)–(e) reproduced from ref. 66 with permission from The Royal Society of Chemistry. (f)–(j) reproduced from ref. 56 with permission from AIP Publishing, Copyright 2011 American Institute of Physics. (k)–(m) reproduced from ref. 71 with permission from Elsevier, Copyright 2016.

by the wall structures and are allowed to form into microtissues; after the formation of the microtissues in the cell culture area, the microtissue as well as the wall structure and the surround channels mimics the microscopic structure in liver tissue called hepatic cords (Figure 3.7(i–j)).⁵⁶

On-chip anchoring techniques are used to provide feasible fixation of bulky tissues which are formed on the microfluidic chips. The anchoring techniques are proposed to solve a general problem in the formation of homogenous bulky tissues such as skins; such tissues are formed by molding a cell-ECM mixture into the cavities of the culture devices. Since the initial concentration of ECM precursor solutions are usually low (high concentrations will result in highly viscous solutions that are difficult to handle and gelate rapidly), the size of the final formed bulky tissues are often smaller than the size of the molding cavities due to shrinkage of ECMs caused by cell–cell and cell–matrix traction forces. To avoid such shrinkages, which will cause the fabricated tissues to detach from the molding devices, anchoring structures are proposed. Vollert *et al.* designed culture devices with standing posts to provide anchoring for the shrinking rod-shaped tissues made up of neonatal rat heart cells and fibrin; the posts that support the fabricated tissues were embedded with perfusable microchannels. With the use of alginate microfibers as a sacrificial template, the fabricated tissues could be cultured with constant perfusion.⁶⁸ Hansen *et al.* also proposed similar approaches by creating standing posts to anchor the engineered heart tissue also based on neonatal rat heart cells and fibrin.⁶⁹ Based on the strategy of combining perfusable posts with sacrificial microchannels, Abaci *et al.* further advanced the techniques by creating arbitrary-shaped sacrificial channels using 3D printing techniques—the whole culture device is designed to fit into tissue culture inserts, allowing stable and sterile culture conditions.⁷⁰ To further unleash the power of 3D printing technology in device prototyping, Mori *et al.* proposed fully 3D printed microfluidic culture device for the creation of vascularized skin equivalent with perfusable microchannels (Figure 3.7(k)); the inlets of the perfusable channels were 3D printed with hook-style anchoring structures for feasible sealing of the perfusable channels (Figure 3.7(l)), in addition, oxygen plasma treatment of the culture device before seeding of the cell-ECM mixture was also found to benefit the quality of the sealing (Figure 3.7(m)).⁷¹ Also based on 3D printing, Morimoto *et al.* proposed methods to enhance the anchoring of cardiomyocytes-based microtissues to culture devices by increasing the number of anchoring posts; in the reported devices, arrays of anchoring posts are densely fabricated using stereolithography to provide feasible fixation of the formed microtissues for further microscopic investigations.⁷²

3.4.1.3 On-chip Construction of Tissue–Tissue Interfaces

Tissue-tissue interfaces, such as the interface between vascular endothelium and surrounding connective tissue and parenchymal cells, are crucial to the function of nearly all organs.⁷³ The most important part of tissue–tissue

interfaces are the endothelial monolayers, which are suitable to be cultured and assayed using traditional dish culture methods and has been investigated intensively. However, the traditional dish culture methods using tissue culture dishes and tissue culture inserts lack the crucial microenvironments as in the native tissue or organ. The application of shear forces and strain forces that is applied to *in vivo* tissue–tissue interfaces in organs such as lungs, guts and intestines is difficult to achieve using traditional culture methods. It is under this context that the reconstruction of tissue–tissue interfaces in microfluidic chips proves its novelty. A representative approach for the construction of tissue–tissue interfaces in microfluidic chips was proposed by Huh *et al.*⁷⁴ In their PDMS-based microfluidic chips, tissue–tissue interfaces were reconstructed by seeding cells on both sides of a porous membrane; such membranes have been massively used as substrate for tissue culture inserts and hence are suitable for cell attachments. In the device proposed by the authors, the porous membrane is sandwiched in between two microfluidic channels so that liquid-to-liquid and liquid-to-gas interfaces could be mimicked by introducing liquid or air samples into the microfluidic channels. In addition, the side walls of the two microfluidic channels are thin and elastic, so that they could be bent upon application of pneumatic pressures to create stretching and/or compressing strains on the porous membrane. The optical transparency of the PDMS channel walls and the porous membranes enables the non-destructive investigation of cell morphologies at high resolution using phase-contrast microscopy as well as fluorescent investigations of cellular components; such visibility could never be achieved in *in vivo* studies. Based on the methodology established in this work, lung-on-a-chip,^{74,75} gut-on-a-chip^{76–78} and other organ-on-a-chip^{79–82} platforms were proposed subsequently. These organ-on-a-chip platforms are very useful in the modeling of organs for pharmacokinetic researches since they can eliminate the amount of animal experiments during the drug developments. In addition, since it is possible to construct these on-chip organs using cells from the patient, precise and customized medical care could be enabled in the near future.

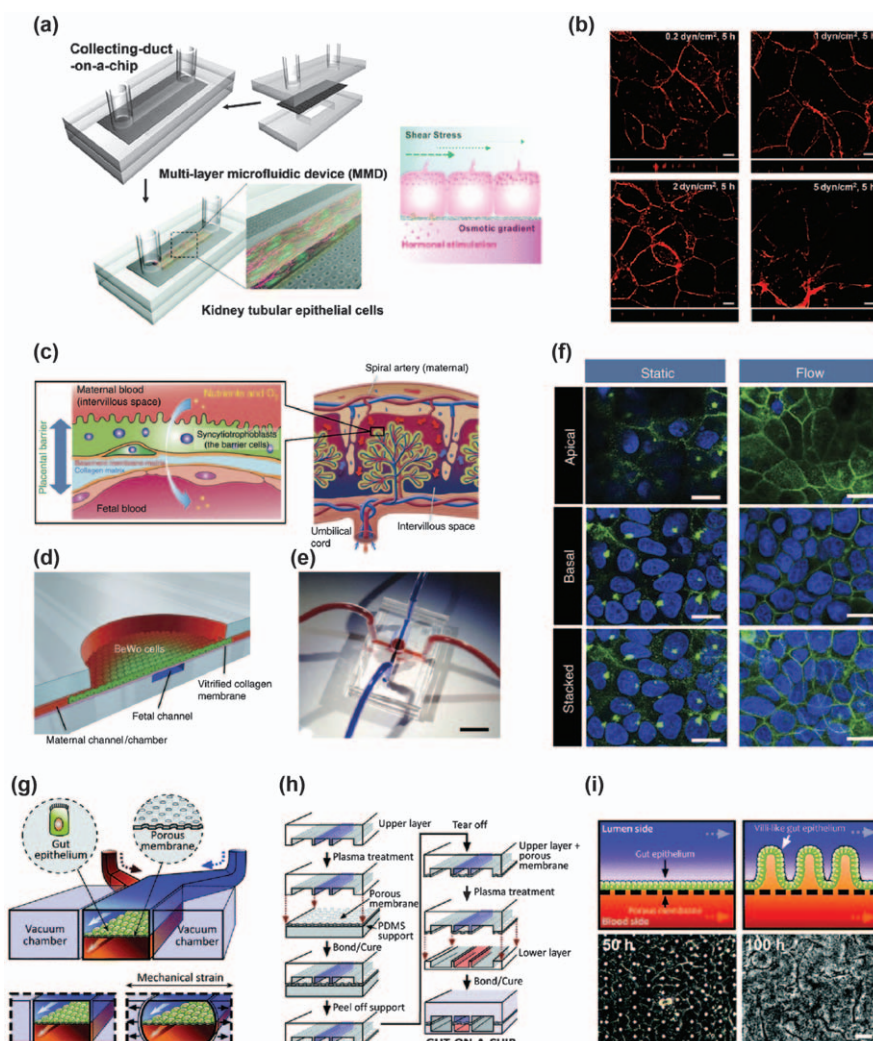
3.4.2 On-chip Tissue Sensing and Stimulation Techniques

With the tissues constructed or installed on the microfluidic chips, the modeling of 3D tissue is ready to go. The modeling of tissue is based on the static measurement of the tissue composition and morphology, as well as the dynamic recording of tissue behaviors and responses in accordance to specific environmental stimulations. The static modeling of tissues has been conducted by biologists and medical doctors through the centuries using the anatomical and histological analysis techniques. However, the anatomy- and histology-based approaches have difficulties in modeling the detailed and precise behavior of the tissues (on a cellular level) under stimulations such as drugs, electrical stimulations, and mechanical stimulations (such as shear forces and strain forces). The emergence of microfluidic technologies

can provide methodologies for the precise stimulation and measurement of tissues, on cellular level. In the following texts, we will show the relative progresses of the on-chip tissue sensing/stimulations platforms.

3.4.2.1 Mechanical Stimulation

In the previous sections, we have shown that deformable microchannels with tissue–tissue interfaces can be designed and fabricated in PDMS-based microfluidic chips. Using these chips, mechanical stimulations such as shear forces and strain forces can be applied to the on-chip tissue–tissue interfaces and the cellular responses to the stimulations can be measured on chips.



Shear force can be applied to tissue–tissue interfaces by generating flows in the microfluidic channels. The magnitude of the forces can be precisely tuned by changing the fluidic inputs. Jang *et al.* proposed a multilayer microfluidic chip by integrating a PDMS microfluidic channel and a porous membrane substrate to culture and analyze the renal tubular cells; taking the *in vivo* tubular environments for the cells as a reference, an optimal fluidic conditions for the cultured cells (*i.e.* fluidic shear stress of 1 dyn cm^{-2}) was verified by enhanced cell polarization, cytoskeletal reorganization, and molecular transport by hormonal stimulations.⁸³ Jang *et al.* then showed that the fluid shear stress and *trans*-epithelial osmotic gradient works with several other factors collectively to exert a profound effect on the AQP2 trafficking in the collecting ducts, which is associated with actin cytoskeletal reorganization in renal tubular epithelial cells (Figure 3.8(a–b)).⁸⁴ Miura *et al.* fabricated a multilayer microfluidic device to analyze material transport through the cells and to observe cellular responses to a broad range of fluid shear stress; the authors demonstrated that fluid shear stress serves as a trigger for microvilli formation in human placental trophoblastic cells (Figure 3.8(c–f)).⁷⁹

Many of the internal organs (such as lung, gut, *etc.*) contain sack-like tubular tissues which consist of elastic outer layers that allow expansions and compressions. Huh *et al.* built a microfluidic chip with porous cell-laden membranes sandwiched in between two microfluidic channels; the channel walls were made of thin layers of PDMS that could be actively deformed with pneumatic infusion and suction. Using the device, they revealed that cyclic mechanical strain accentuates toxic and inflammatory responses of the lung to silica nanoparticles.⁷⁴ Kim *et al.* used the similar devices and reconstructed human gut as gut-on-a-chip; by ceasing peristalsis-like motions while maintaining luminal flow, a lack of epithelial deformation was shown to trigger bacterial overgrowth similar to that observed in patients with ileus and inflammatory bowel disease.⁷⁷ Kim *et al.* also showed that in a human gut-on-a-chip, the development of columnar epithelium with rapid polarization under

Figure 3.8 Mechanical stimulations for tissues on-chip. (a) Multilayer microfluidic device with kidney tubular epithelial sheets rebuilt on-chip and (b) the effects of flow velocity on F-actin depolymerization showing that fluid shear stress larger than 1 dyn cm^{-2} induced the full depolymerization of F-actin.⁸⁴ (c–e) Concepts and implementation of a placenta-on-a-chip to analyze material transport through the cells; (f) shows that fluid shear stress serves as a trigger for microvilli formation in human placental trophoblastic cells.⁷⁹ (g–h) Concept and fabrication method of the gut-on-a-chip device⁷⁶ and (i) showing that the villus differentiation of human intestinal cells (Caco-2 cells) can be induced by exposing the cultured cells to physiological peristalsis-like motions and liquid flow.⁷⁸ (a) and (b) reproduced from ref. 84 with permission from The Royal Society of Chemistry. (c)–(f) reproduced from ref. 79, <https://doi.org/10.1038/ncomms9871>, under the terms of the CC BY 4.0 License, <https://creativecommons.org/licenses/by/4.0/>. (g) and (h) reproduced from ref. 76 with permission from The Royal Society of Chemistry. (i) reproduced from ref. 78 with permission from The Royal Society of Chemistry.

a low shear stress of 0.02 dyn cm^{-2} and cyclic strain of 10% at 0.15 Hz which mimics physiological peristaltic motions (Figure 3.8(g–h)).⁷⁶ Kim *et al.* then went on to demonstrate that the villus differentiation of human intestinal cells (Caco-2 cells) can be induced by exposing the cultured cells to physiological peristalsis-like motions and liquid flow (Figure 3.8(i)).⁷⁸

3.4.2.2 Chemical Sensing and Stimulation

Another major type of tissue signals, besides the previously introduced mechanical type, is the chemical type of tissue signals. Chemical sensing- or stimulation-based drug assays are at the center of the state-of-the-art pharmaceutical industry. The microfluidic chip-based platform is advantageous in the following three aspects: first, since most of the microfluidics are made of optically transparent and non-toxic materials, the stable investigation of cells using optical microscopy is possible; second, the manipulation of extremely small amounts of liquid using microfluidic methods allows precise sampling of precious substances secreted by cells, as well as the deduction of the usage of stimulating chemicals used for the assays; third, the reconstructed tissues in microfluidic chips mimic micro-scale tissue architecture and provide tissue–tissue interfaces that are closer to native tissues compared to traditional tissue cultures using culture dishes. Recently, organ-on-a-chip start-up companies have begun to spawn from academic research to fill this commercial space and are attracting investment to transform the drug discovery industry.⁸⁵

Here we give a brief summary of representative academic works related to chemical sensing and stimulation on-chip. Some works concentrated on the online assay of the mono-cultured single tissue or co-cultured tissues on chips. Imura *et al.* built bioassay microchips upon which three different tissues are reconstructed and connected; using the chip, the activities of anticancer agents and estrogen-like substances were successfully assayed (Figure 3.9 (a–d)).⁸⁶ Chen *et al.* designed and implemented a multi-microphysiological systems (MPS) platform hosting immune-competent gut and liver models and studied human gut–liver tissue interactions through chemical signals under normal and inflammatory contexts (Figure 3.9(e–h)).⁸⁷

The next few works concentrate on the barrier function of endothelial cells along the tissue–tissue interfaces. Mori *et al.* developed skin-on-a-chip devices and managed to create perfusable channels in the skin (with epidermis on the top) with endothelial cells lining its inner surface; using the device, the authors demonstrated the measurement of test molecule permeation from the epidermal layer into the vascular channels.⁷¹ Abaci *et al.* constructed thick skin (with epidermis on the top) tissues on top of a porous membrane and attached perfusable channels down beneath the membrane; they used the chip with a transdermal transport model to analyze skin barrier functions (Figure 3.9(i)).⁸⁸ Wang *et al.* developed a microfluidic blood–brain barrier (BBB) model that is capable of mimicking *in vivo* BBB characteristics and performed drug permeability studies using large

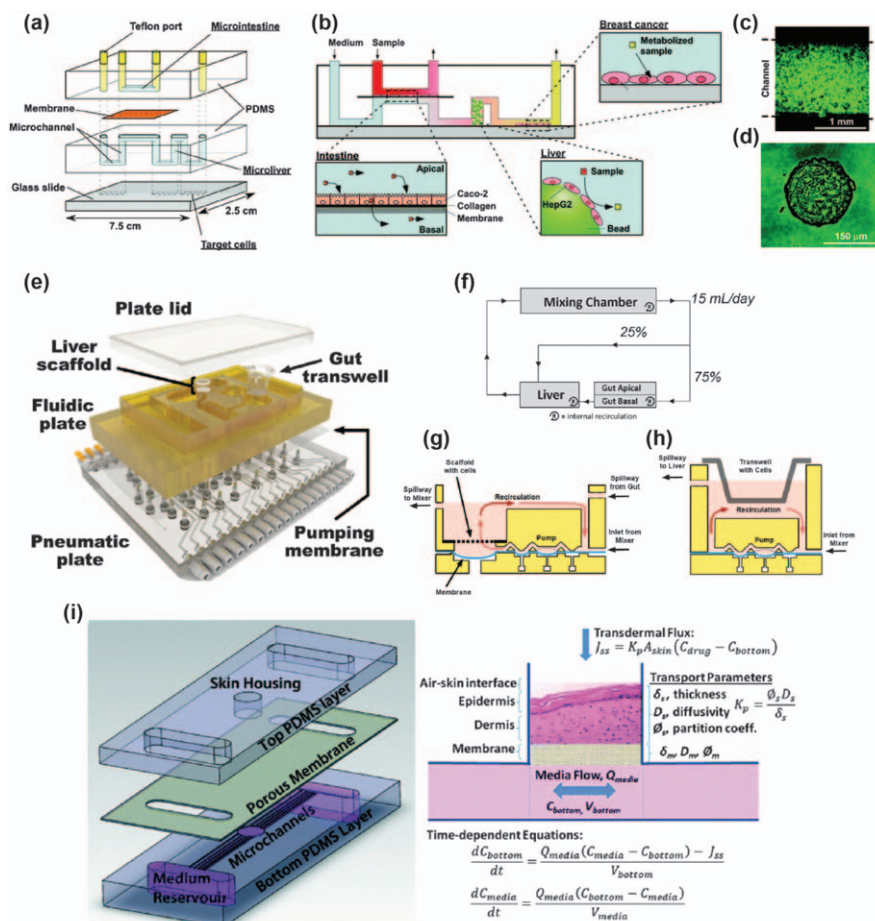
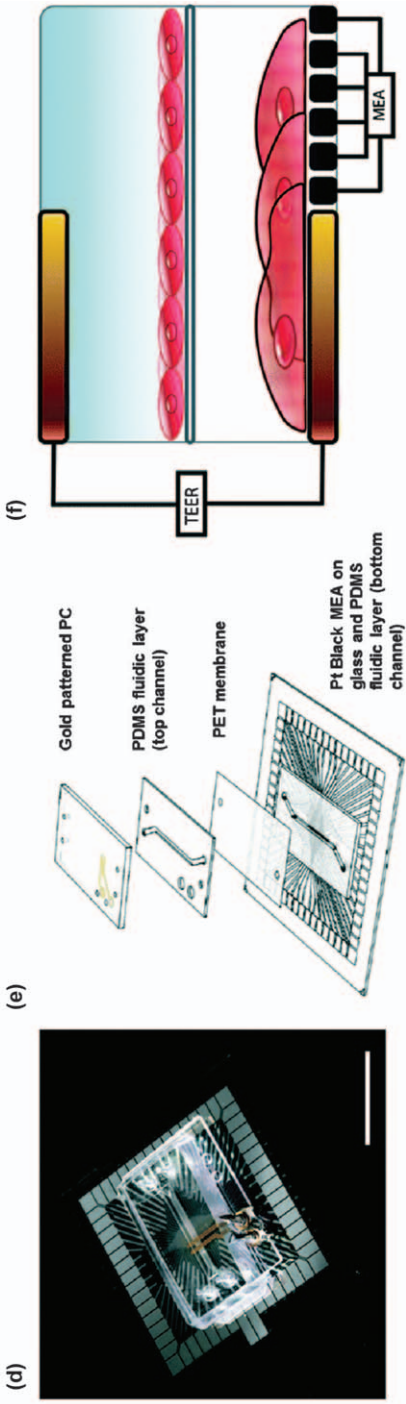
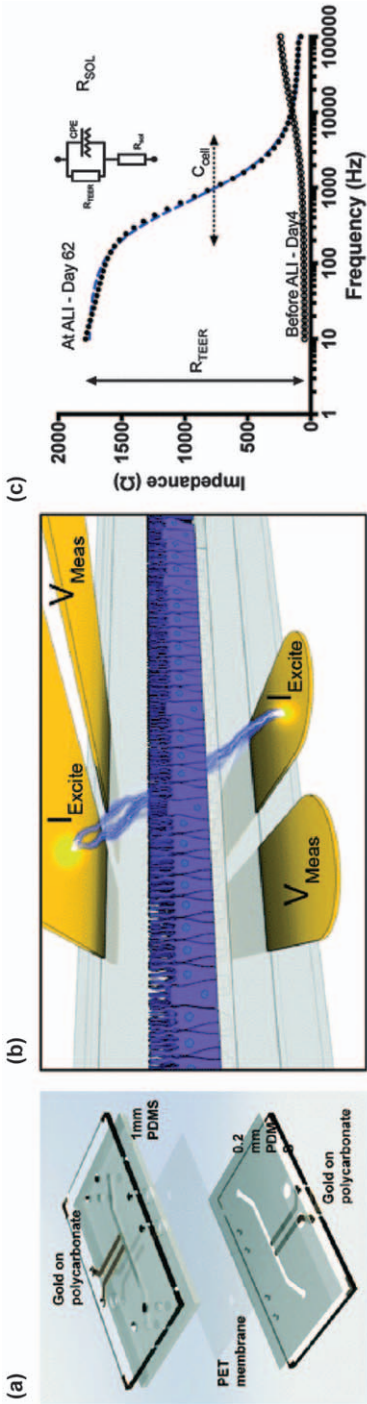


Figure 3.9 Chemical sensing and stimulation for tissues on chip. (a–b) Fabrication method and concept illustration of the microfluidic platform used for the assessment of intestinal absorption, hepatic metabolism, and bioactivity; (c–d) shows Caco-2 cells cultured on a membrane support in a microchamber stained with calcein-AM and a Cytodex bead with HepG2 cells on its surface.⁸⁶ (e) A multi-microphysiological systems (MPS) platform hosting immune-competent gut and liver models for the study on human gut–liver tissue interactions and (f–h) its operation schemes and the realization method of recirculating flows.⁸⁷ (i) Microfluidic chip with thick skin (with epidermis on the top) tissues on top of a porous membrane and perfusable channels down beneath the membrane; the chip is used with the transdermal transport model to analysis the skin barrier functions.⁸⁸ (a)–(d) reproduced from ref. 86 with permission from American Chemical Society, Copyright 2010. (e)–(h) reproduced from ref. 87, <https://doi.org/10.1002/bit.26370>, under the terms of the CC BY 4.0 license, <https://creativecommons.org/licenses/by/4.0/>. (i) reproduced from ref. 88 with permission from The Royal Society of Chemistry.



molecules (FITC-dextran) and model drugs (caffeine, cimetidine, and doxorubicin).⁸²

3.4.2.3 Electrical Sensing and Stimulation

The last major type of tissue signals are the electrical ones. The related works are categorized into two subcategories: simulation and sensing.

On the stimulation side, new advances in tissue engineering are being made through the application of different types of electrical stimuli to influence cell proliferation and differentiation.^{89,90} Specific cells such as neurons and cardiomyocytes can be stimulated using electrical signals to guide their growth direction, motion, and movements; such electrical-to-mechanical signal transformations are useful for drug screening⁷² and biohybrid robotics.^{91–93} Pavesi *et al.* designed and fabricated a microfluidic chip imitating the lung-on-a-chip design and managed to incorporate electrodes on-chip; bone marrow mesenchymal stem cell-based tissue sheets were reconstructed on-chip and cell orientation was found to be related to electromechanical stimulations.⁹⁴ Linda *et al.* proposed a 3D printed heart-on-a-chip device with integrated strain gauges for direct readout of tissue contractile strength; using the device, the authors performed multiplex drug-dose experiments and studies of functional maturation of cardiac tissue.⁹⁵

On the sensing side, the function of tissue barriers can be probed by measuring the *trans*-epithelial electrical resistance (TEER); healthy tissue barriers tend to have higher TEERs in comparison with injured tissue barriers. Ramadan *et al.* proposed a microfluidic chip with electrodes as a miniaturized immune competent *in vitro* model of human skin; TEER measurement data indicated that the tight junction formation could be improved with dynamic perfusion of culture media.⁹⁶ Henry *et al.* proposed a microfluidic organ chip design that contains embedded electrodes; they applied the devices to construct both human lung airway chips lined by fully differentiated mucociliary human airway epitheliums and human gut chips lined by intestinal epithelial cells. The formation and disruption of barrier functions were monitored by measuring TEER (Figure 3.10(a–c)).⁹⁷ Maoz *et al.* improved the device designs

Figure 3.10 Electrical sensing/stimulation for tissues on chip. (a) The microfluidic chip with the integrated electrodes and (b) the scheme of *trans*-epithelial electrical resistance (TEER) measurement and (c) the impedance spectra recorded before air–liquid interface (ALI) and after full differentiation at ALI.⁹⁷ (d) both multi-electrode arrays (MEAs) and electrodes for TEER measurements; (e–f) the fabrication method of the microfluidic chips with sophisticated electrodes, and the use of the TEER–MEA chip to simultaneously study both the integrity of the endothelium (the upper cells in the illustration) and the electrical activity of heart cardiomyocytes (the bottom cells in the illustration).⁹⁸ (a)–(c) reproduced from ref. 97 with permission from The Royal Society of Chemistry. (d)–(f) reproduced from ref. 98 with permission from The Royal Society of Chemistry.

by integrating both multi-electrode arrays (MEAs) and electrodes for TEER measurements to provide multifunctional, real-time sensing capabilities (Figure 3.10(d–f)).⁹⁸

3.5 Conclusions

In this chapter, we introduced the basic concepts of microfluidics and its applications in the field of biofabrication and 3D tissue modeling. We categorized the current works into two major categories: tissue-off-chip platforms and tissue-on-chip platforms. The tissue-off-chip platforms concentrate on the fabrication side of microtissues with high throughput. The tissue-on-chip platforms concentrate on the modeling of minimal tissues (*i.e.* the greatly simplified tissues) using microfluidic chips to emulate macro tissues, organs or even human beings. We believe the converging of knowledge and know-how on both ends will finally lead to the rapid fabrication of functional organs to boost the field of tissue engineering and regenerative medicine.

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CHAPTER 4

Computational Design and Modeling of Linear and Nonlinear Elastic Tissue Engineering Scaffold Triply Periodic Minimal Surface (TPMS) Porous Architecture

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4.1 Introduction

Butler *et al.* first proposed the concept of “functional tissue engineering” as a set of design principles for tissue engineering in 2000.¹ Two of the six

principles related to design required to achieve the desired mechanical properties for tissue engineering constructs are:

1. “The mechanical properties of the native tissues must be established for subfailure and failure conditions”
2. “A subset of these mechanical properties must be selected and prioritized”

Guilak *et al.* extended these conditions in a follow-up article in 2014² to characterize mechanical properties across physical scales and to address the design and development of biomaterials and scaffolds with specific mechanical properties to serve as a foundation for tissue engineering constructs:

1. “Understanding the biomechanical properties of native and repair tissues across all geometric scales”
2. “Prioritization of specific mechanical properties as design parameters for biomaterial scaffolds and engineered tissues”
3. “Development of biomaterials and scaffold with prescribed biomechanical properties”

These principles highlighted the need to understand native tissue mechanical properties and the need to design scaffolds with controlled mechanical properties characterized in relation to native tissue mechanical properties. Furthermore, Butler *et al.*¹ highlighted that the mechanical properties to match as scaffold design targets include anisotropy, linear elasticity, nonlinear elasticity, nonlinear viscoelasticity, and nonlinear permeability. Anisotropy, linear elasticity, and linear permeability are relevant to bone tissue engineering while anisotropy, nonlinear elasticity, nonlinear viscoelasticity, and nonlinear permeability (deformation dependent permeability) are most relevant for soft tissue engineering.

The question becomes, as noted by Butler *et al.*¹ and Guilak *et al.*,² which are the most relevant mechanical properties to match and what is the scale at which they are matched. As a starting point, we choose to match mechanical properties at the most macroscopic, termed effective, scaffold scale. Referencing Harrigan *et al.*,³ the most macroscopic continuum assumption is valid over 3 to 5 characteristic microscopic features. In their example of cancellous bone, this was equivalent to 3 to 5 trabeculae plus marrow space. For porous scaffolds, an equivalent scale would be 3 to 5 pores plus scaffold material features.

In terms of mechanical properties, a starting basis is to design for isotropic and anisotropic linear elastic mechanical properties for hard tissue engineering scaffolds and isotropic and anisotropic nonlinear elastic mechanical properties for soft tissue engineering scaffolds. In terms of mass transport properties, isotropic and anisotropic permeability is a good starting basis for designing both hard and soft tissue scaffolds. Thus, a baseline premise for hard tissue engineering scaffold design is arranging base

scaffold material and pore structure to achieve the desired macroscopic continuum permeability and linear elastic properties. A baseline premise for soft tissue engineering scaffold design is arranging scaffold material and pore structure to achieve the desired macroscopic continuum permeability and nonlinear elastic properties.

Hard tissue engineering scaffold design has been widely studied for over 20 years. Both forward and inverse techniques generate hard tissue engineering scaffold design. Forward techniques first postulate a scaffold pore architecture and then compute effective linear elastic and permeability constants under a variety of boundary conditions on the Representative Volume Element (RVE). This architecture can be generated in a variety of forms including geometric forms like spheres and cylinders, or a class of structures known as Triply Periodic Minimal Surfaces (TPMS).

Periodic displacement boundary conditions are utilized in classic homogenization theory, and it has been demonstrated that these boundary conditions lead to effective elastic property estimates in between those of uniform displacement or uniform traction boundary conditions.^{4,5} Pahr and Zysset⁶ proposed the use of mixed displacement and traction boundary conditions for estimating effective elastic properties, suggesting that these boundary conditions could provide better effective elasticity estimates for RVEs that are not periodic or statistically homogeneous. The advantage of the forward technique is that controlled variations in architecture can be made to evaluate the effect of these variations on mechanical properties and these architectures can be tailored for additive manufacturing (*i.e.* 3D Printing).

The disadvantage of the forward design technique is that reaching target effective elastic properties for design is a trial and error process. Furthermore, the scaffold architecture that can achieve effective elastic property design targets may not be obvious. The inverse design process, initially proposed by Sigmund,^{7,8} postulates target effective elastic properties and then uses a topology optimization approach to iteratively update local material densities to produce a final architecture that generates the desired effective elastic properties. A RVE homogenization problem is solved at each optimization iteration step. This approach has been extended to multiphysics problems to design structures simultaneously for effective elasticity and effective mass transport (*i.e.* effective permeability or effective diffusivity) properties.^{9–13} The advantage of the inverse method is the ability to generate structures that could not be a priori conceived using the forward technique. The disadvantage of the inverse technique is that structures can be generated that are not readily manufactured by 3D printing techniques. However, such techniques have been used to produce bone tissue engineering scaffolds *via* 3D printing.^{5,11,14}

Despite the significant advances in estimating scaffold properties based on architectural designs, a number of gaps remain regarding the choice of boundary conditions. First, most computational simulations are compared to uniaxial compression experiments in which a sample is compressed through contact with a, by comparison, rigid platen, with the remaining

sides subject to a zero traction boundary condition. It is uncertain how these contact conditions relate to the assumed RVE boundary conditions, which are postulated to exist on the interior of the architecture, a sufficient distance from the sample contact and zero traction boundary conditions. Second, effective property estimates are nearly always linear estimates. However, as noted in the functional tissue engineering reviews^{1,2} the ability to design and interpret the behavior of scaffolds made from nonlinear materials is needed. In that case, two significant questions arise. The first is how to predict effective nonlinear elastic properties based on nonlinear scaffold base properties and architecture. Given that nonlinear analysis is often difficult to achieve, the second question is whether simpler linear elastic homogenization analyses can be used to estimate or at least bound effective nonlinear elastic properties.

The goal of this chapter is to begin addressing the three questions posed in the preceding paragraph, namely:

1. What is the relationship of effective properties estimated by rigid contact simulations to effective properties estimated by classic periodic and mixed homogenization boundary conditions?
2. How do scaffold architecture and nonlinear material properties relate to effective nonlinear elastic properties?
3. Can effective linear elastic results be used to estimate or at least bound nonlinear effective elastic properties for the same architecture?

To address these questions, nonlinear finite element simulations were performed using the nonlinear finite element code FEBio^{15,16} utilizing both linear and nonlinear elastic base scaffold properties with both rigid and deformable contact to simulate typical uniaxial experimental compression tests. The base scaffold architecture was the TPMS P schwartz structure generated using an image base technique.¹⁷

4.2 Methods

The TPMS P structure¹⁷ was generated from Fourier series nodal approximation equation: $\cos X + \cos Y + \cos Z = 0$ using an image-based approach.

In this approach, the equation is re-written as $\cos\left(2\pi\frac{x}{X_{\text{unit cell}}}\right) + \cos\left(2\pi\frac{y}{Y_{\text{unit cell}}}\right) + \cos\left(2\pi\frac{z}{Z_{\text{unit cell}}}\right)$ where $X_{\text{unit cell}}$, $Y_{\text{unit cell}}$, and $Z_{\text{unit cell}}$ denote the size of a repeated mathematical unit cell in the x , y and z directions, respectively. The minimum and maximum values are then normalized to an 8-bit density scale of 0 to 255. This allows the TPMS structure to be represented as 3D voxel data that based on a threshold will represent volume fractions between 0 to 1. The resulting image data can be read into a variety of programs including SimplewareTM (Synopsys) or Mimics/3-maticsTM

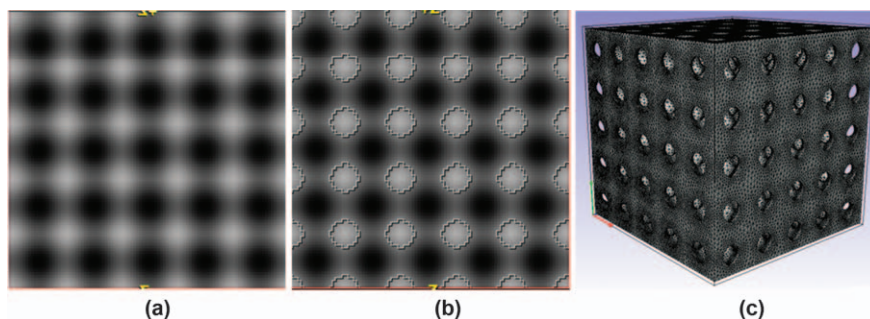


Figure 4.1 Creation of finite element mesh for P Schwarz TPMS architecture from image-design data. (a) Initial greyscale image slice of structure. (b) Outline of mask generated by creating a threshold at 50% volume structure. (c) Resulting 3D 10-node tetrahedral finite element model generated in Simpleware™ from mask.

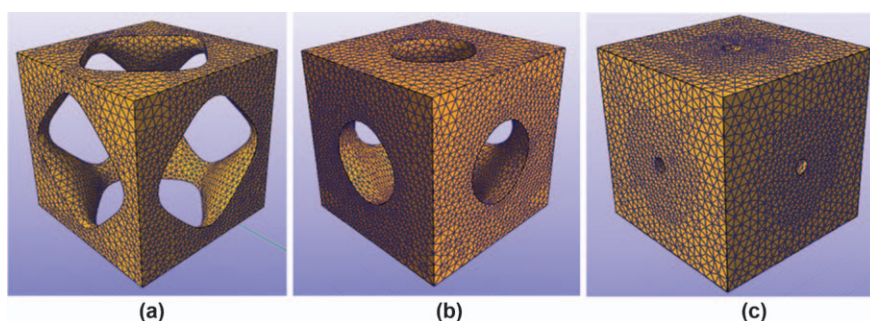


Figure 4.2 Examples of P Schwarz porous architecture in $1 \times 1 \times 1$ unit cell size for (a) 25% volume fraction; (b) 50% volume fraction; and (c) 75% volume fraction.

(Materialise), thresholded, cropped to create scaffolds containing different numbers of unit cells, and then meshed using 10-node tetrahedral elements. Figure 4.1 illustrates the process of going from initial to processed image data to final mesh in Simpleware™.

The approach in Figure 4.1 was used to generate P Schwarz TPMS architectures with 25%, 50% and 75% volume fraction (Figure 4.2).

These architectures were generated in $1 \times 1 \times 1$, $2 \times 2 \times 2$, $3 \times 3 \times 3$, $4 \times 4 \times 4$, and $5 \times 5 \times 5$ unit cell sizes, as it has been shown in previous studies that increasing unit cell numbers in under constant displacement (Dirichlet) or constant stress (Neumann) boundary conditions converge to periodic homogenization predictions from a single unit cell.⁵

P Schwarz structures with different volume fractions created by thresholding in Simpleware™ were exported in Abaqus.inp format and imported into PreView, the FEBio pre-processing program. Rigid platens were generated, contact surfaces between the scaffold architecture and rigid platens were defined, and displacement conditions defined to prevent rigid

body motion. Linear elastic material properties were defined to represent polycaprolactone (PCL) with isotropic elastic properties including Young's modulus $E=290$ MPa, and Poisson's ratio $\nu=0.3$. Nonlinear elastic material properties were chosen to match poly(glycerol sebacate) (PGS), which has been previously characterized as a Neo-Hookean, incompressible nonlinear elastic material with a strain energy function W defined as: $W=c_1(C_{11}+C_{22}+C_{33}-3)$ where c_1 is a model coefficient and C_{11} , C_{22} , and C_{33} are the normal components of the right Cauchy deformation tensor.

All FEBio models were subject to simulated compression between two rigid platens, with nonlinear contact conditions defined at the platen specimen interface. To examine convergence with increasing number of unit cells, models with $1\times1\times1$, $2\times2\times2$, $3\times3\times3$, $4\times4\times4$, and $5\times5\times5$ unit cells where analysed under compression at 5% strain for the PCL model and 20% strain for the nonlinear elastic PGS model (Figure 4.3).

The resulting reaction forces from scaffold architecture compression for each model were then used as input to fit material coefficients for the same constitutive models (*i.e.* isotropic linear elastic or Neo-Hookean nonlinear elastic) for homogenous solid models under the same compression contact boundary conditions. This result gives the linear or nonlinear elastic coefficients for an effective material with the same average mechanical behavior as the porous scaffolds. The parameter optimization routine in FEBio was used to determine the effective constitutive model coefficients giving the best fit between the porous scaffold average reaction force and effective material average reaction force in a least squares sense. Finally, single unit cell models were run using both traditional periodic homogenization^{4,5} and mixed boundary homogenization conditions⁶ assuming linear elasticity on the single unit cell model. These homogenization estimates of effective elasticity were compared to effective linear and nonlinear elastic properties calculated from the contact analyses shown in Figure 4.2.

Finally, a goal of this chapter is to compare effective linear elastic and nonlinear elastic behavior computed using these P Schwarz architecture designs of differing volume fraction to reported soft tissue material properties. This task is complicated by the fact that soft tissue nonlinear elastic behavior is computed using a wide range of nonlinear elastic constitutive models derived from a wide range of isotropic and anisotropic strain energy

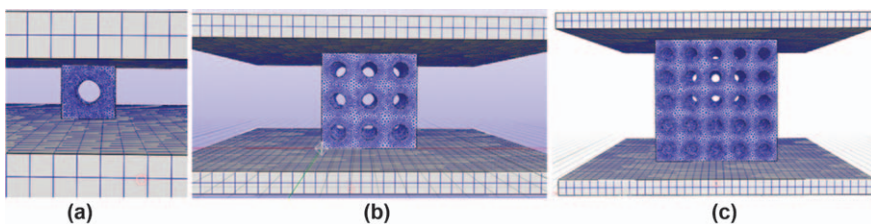


Figure 4.3 FEBio models for simulation of 50% porous PCL and PGS scaffolds having (a) $1\times1\times1$ to (b) $3\times3\times3$ to (c) $5\times5\times5$ unit cells.

functions. To create apples to apples comparison, a range of soft tissue nonlinear elastic behaviors were modeled by using strain energy functions and associated constants in the literature modeled as a block under contact compression. The resulting stretch ratio λ in the compression direction was computed as the initial height minus the displacement divided by the initial height. The finite strain component was then computed as $E = \frac{1}{2}(\lambda^2 - 1)$. The 1st Piola–Kirchhoff (PK) stress P in compression was computed as the reaction force F divide by the initial area A^0 : $P = \frac{F}{A^0}$. The 1st PK stress was converted to the 2nd PK stress S by: $S = \frac{P}{\lambda}$. The 2nd PK stress S versus finite strain E was plotted for both soft tissues and scaffold architectures. The tissues and strain energy functions used for comparison are detailed in Table 4.1.

4.3 Results

A total of 29 nonlinear contact analyses (Figure 4.2) with $1 \times 1 \times 1$, $2 \times 2 \times 2$, $3 \times 3 \times 3$, $4 \times 4 \times 4$, and $5 \times 5 \times 5$ unit cell configurations were performed with both an isotropic linear elastic material and a nonlinear elastic incompressible Neo-Hookean material simulating the biopolymer PGS.¹⁸ The linear elastic PCL material was subject to 5% compressive strain and the nonlinear PGS material was subject to 20% compressive strain. The reaction forces on the rigid platens were used as input to the parameter optimization module in which the effective constitutive coefficients of a homogeneous block model (either linear elastic or nonlinear Neo-Hookean) were adjusted to provide to the best least squares fit to the original model (Figure 4.2) reaction forces. The effective constitutive coefficient ratio to the original homogenous constitutive coefficient was calculated as a percentage. Thus, solid material would have a ratio of 100% and the ratios are expected to decrease as the scaffold architecture volume decreases with increasing porosity. This constitutive ratio percentage was then compared to both periodic displacement linear elastic homogenization estimates on a single unit cell^{4,5} and mixed boundary linear elastic homogenization estimates on a single unit cell.⁶ Both periodic and mixed boundary homogenization estimates were made using Simpleware™ for 25%, 50% and 75% volume fraction P Schwarz architectures.

Figure 4.4a shows the percentage difference between multi-cell contact linear elastic analyses and the periodic boundary condition (BC) single cell estimates of effective linear elastic Young's modulus for 25%, 50%, and 75% volume fraction P Schwarz TPMS architecture. Figure 4.4b shows the percent difference between multi-cell contact nonlinear Neo-Hookean PGS analyses and the periodic BC single cell estimates of effective linear elastic Young's modulus for 25%, 50%, and 75% volume fraction P Schwarz TPMS architecture.

The results demonstrate larger differences between the contact analysis and periodic BC homogenization estimates as porosity increases. Classic Voight

Table 4.1 Soft tissues used to tissue regeneration and their associated mechanical characterization using nonlinear strain energy functions.

Tissue	Species	Strain energy function	Material coefficients	Reference
Tracheal Cartilage	Human	$W = c_1(I^1 - 3)$	c_1	19
Tracheal Cartilage	Sheep	$W = \frac{c_1}{c_2^2}(\lambda_1^{c_2} + \lambda_2^{c_2} + \lambda_3^{c_2} - 3)$	c_1, c_2	20
Auricular Cartilage	Human	$W = c_1(I^1 - 3) + c_2(I^2 - 3)$	c_1, c_2	21
Articular Cartilage	Human	$W = c_1 \frac{1}{2}(I^1 - 3) + c_1 \frac{1}{20c_2}([I^1]^2 - 9)$ $+ c_1 \frac{11}{1060c_2^2}([I^1]^3 - 27)$ $+ c_1 \frac{19}{7000c_2^3}([I^1]^4 - 81)$ $+ c_1 \frac{19}{7000c_2^4}([I^1]^5 - 243)$	c_1, c_2	22
Skin	Human	$W = \frac{c_1}{c_2^2}(\lambda_1^{c_2} + \lambda_2^{c_2} + \lambda_3^{c_2} - 3)$	c_1, c_2	23
Skin	Human	$W = \frac{c_1}{c_2^2}(\lambda_1^{c_2} + \lambda_2^{c_2} + \lambda_3^{c_2} - 3)$	c_1, c_2	24

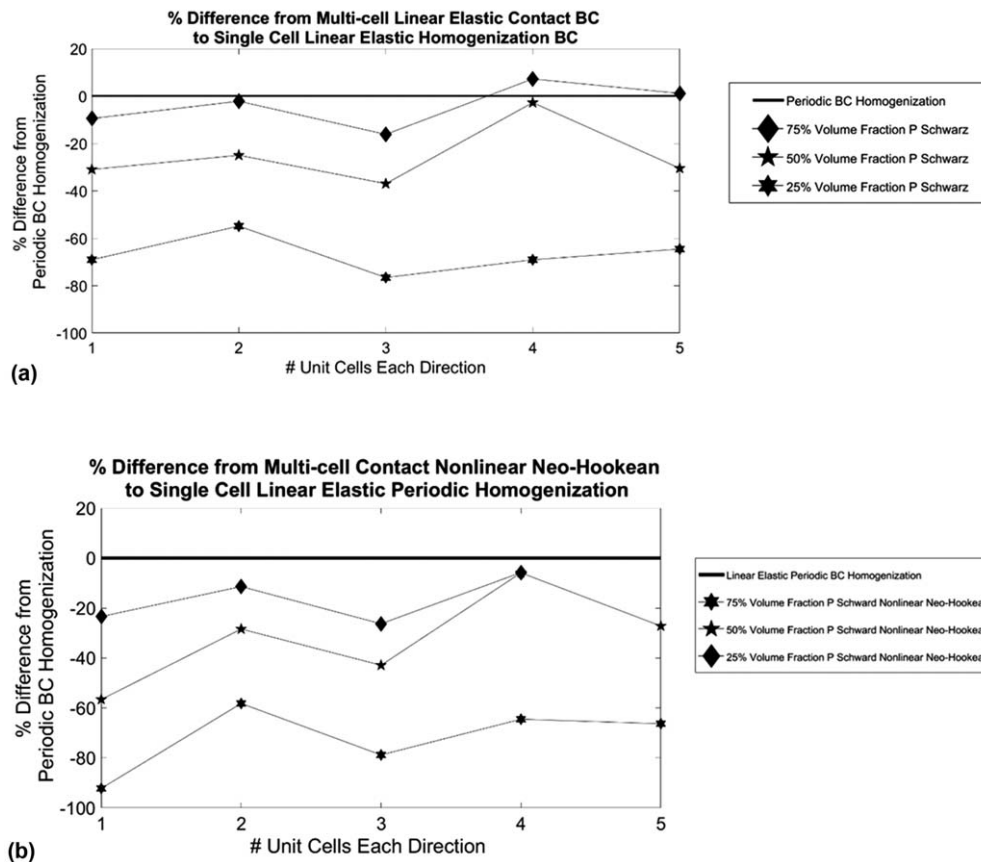


Figure 4.4 Percentage difference between linear elastic periodic BC homogenization and (a) linear elastic multi-cell contact analysis and (b) nonlinear Neo-Hookean multi-cell contact analysis for 25%, 50% and 75% volume fraction P Schwarz porous scaffold architecture.

bounds (volume fraction times material coefficients) were greater than homogenization estimates by 13% (75% volume fraction), 32% (50% volume fraction), and 42% (75% volume fraction). Thus, Voight bounds are an upper bound on contact analysis for both the linear elastic and nonlinear elastic materials. However, the contact analysis results do not demonstrate a monotonic convergence to the single cell periodic BC homogenization results.

Figure 4.5a shows the percentage difference between multi-cell contact linear elastic analyses and the mixed BC single cell estimates of effective linear elastic Young's modulus for 25%, 50%, and 75% volume fraction P Schwarz TPMS architecture. Figure 4.5b shows the percent difference between multi-cell contact nonlinear Neo-Hookean PGS analyses and the mixed BC single cell estimates of effective linear elastic Young's modulus for 25%, 50%, and 75% volume fraction P Schwarz TPMS architecture.

The results again demonstrate larger differences between the contact analysis and periodic BC homogenization estimates as porosity increases. The contact analysis results again did not demonstrate a monotonic convergence to the single cell periodic BC homogenization results. However, the mixed BC homogenization results showed less difference to the multi-cell contact analysis than the periodic BC homogenization results.

The ultimate purpose of this design approach is to determine what effective nonlinear elastic and permeability properties can be achieved from a given base material fabricated in a specific porous architecture design, and how these effective properties relate to actual tissue nonlinear properties. Second PK stress *versus* finite strain were plotted for tissues (Table 4.1), PCL P Schwarz architectures (25%, 50%, 75%, and 100% volume fraction), and PGS P Schwarz architectures (25%, 50%, 75%, and 100% volume fraction). The scaffold only plot (Figure 4.6) shows the intuitive relationships between the stiffer linear elastic PCL and the nonlinear elastic PGS with varying volume fractions of P Schwarz architecture.

Figure 4.7 illustrates the lower volume fraction PCL scaffold and all PGS scaffolds *versus* tracheal, auricular, and articular cartilage.

Figure 4.8 illustrates stress-strain behavior of lower volume fraction PGS scaffolds *versus* skin. In this case, even porous PGS exhibits stiffer nonlinear behavior than skin. Decreasing PGS volume fraction decreases not only the stiffness but the degree of PGS nonlinearity.

4.4 Discussion

The purpose of this chapter was to answer the following questions: (1) how do contact boundary condition estimates of homogenized elastic scaffold properties relate to classic periodic and mixed single cell homogenization estimates? (2) how do scaffold nonlinear material and architecture relate to effective nonlinear elastic properties? and (3) can linear elastic estimates of homogenized effective properties estimate or at least bound nonlinear homogenized effective elastic properties? With regard to question 1, the contact boundary conditions exhibited similar bounding behaviors to

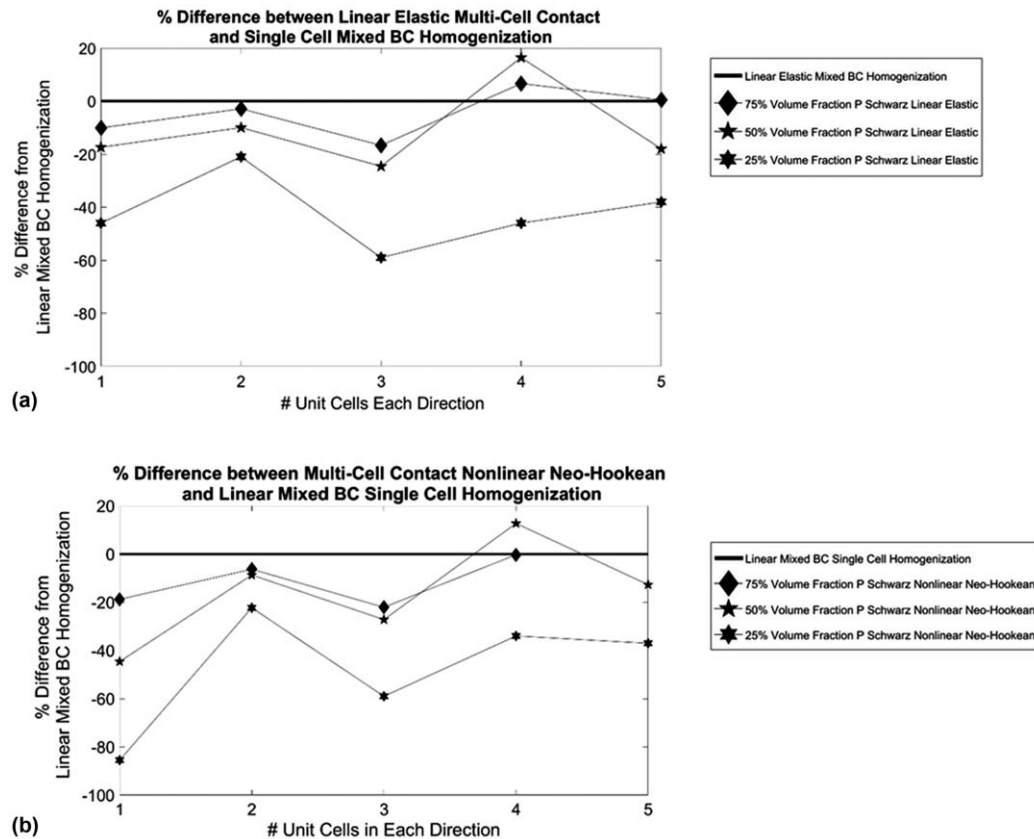


Figure 4.5 Percent difference between linear elastic mixed BC homogenization and (a) linear elastic multi-cell contact analysis and (b) nonlinear Neo-Hookean multi-cell contact analysis for 25%, 50% and 75% volume fraction P Schwarz porous scaffold architecture.

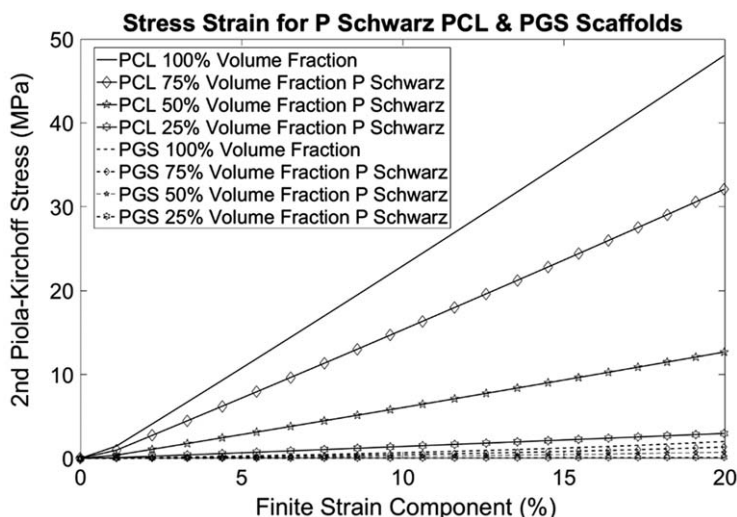


Figure 4.6 Stress-strain plots for PCL and PGS P Schwarz scaffold architectures of varying volume fractions. PCL demonstrates much greater stiffness. The PCL 25% volume fraction approaches the 100% volume fraction PGS material. Note PGS exhibits nonlinear behavior but due to the scale of the plots the PGS stress-strain plots appear linear.

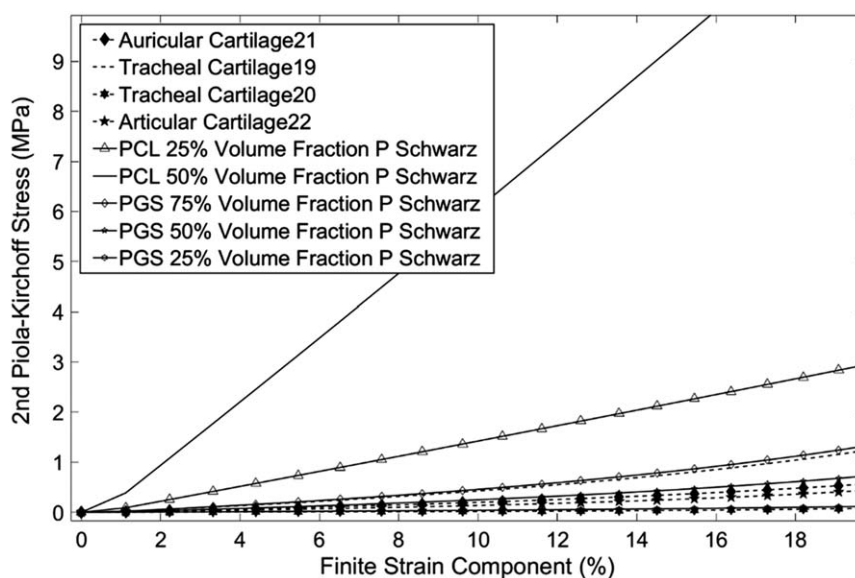


Figure 4.7 Stress-strain behavior of PCL and PGS scaffolds *versus* stress-strain behavior of tracheal, auricular and articular cartilage. PCL scaffolds (25% and 50% volume fraction) exhibit stiffer behavior than reported cartilage values. PGS scaffolds exhibit stress-strain behavior within the range of cartilage tissues.

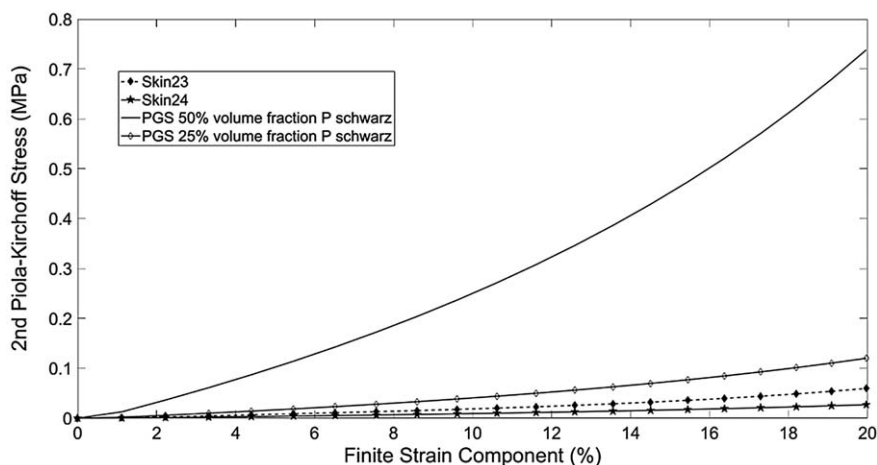


Figure 4.8 Nonlinear stress-strain behavior of skin *versus* porous PGS. PGS P Schwarz scaffolds of both 25% and 50% volume fraction are stiffer than reported skin values.^{23,24}

non-contact Dirichlet (displacement) boundary conditions demonstrated by Coelho *et al.*⁵ Furthermore, linear elastic effective moduli estimates for the 50% P Schwarz scaffolds were similar to those reported previously for Dirichlet boundary conditions.²⁵ The classic periodic homogenization results provided an upper bound on the contact analyses in most cases. The periodic mixed boundary conditions⁶ exhibited less difference than the classic periodic boundary conditions to both linear elastic and nonlinear elastic. However, in neither case did the contact analysis result show monotonic convergence to homogenization results as previously demonstrated.⁵ This lack of convergence could result from greater variations in contact analyses compared to Dirichlet boundary conditions. Furthermore, the presence of a rigid plate could also affect the results.

With regard to question 2 for the current Neo-Hookean materials, nonlinear scaffold material and architecture showed similar trends in relationship to effective nonlinear properties as did linear elastic materials. Namely, with increasing porosity of scaffolds made from nonlinear elastic materials decreases the nonlinear effective stiffness of these scaffolds. Furthermore, the degree of nonlinearity decreases as porosity increases, which has been noted previously in nonlinear elastomeric scaffolds.^{18,26} A caveat to this result is the finding that nonlinear materials defined in porous structures can exhibit material instabilities not seen with linear materials, a phenomenon described in the composite homogenization literature as loss of ellipticity in the macroscopic effective constitutive model due to instability at the microscopic scale.²⁷ This phenomenon was seen in the most porous 25% volume fraction PGS scaffolds, where there is a loss of periodic structure and a large increase in deformation without any increase in force (Figure 4.9).

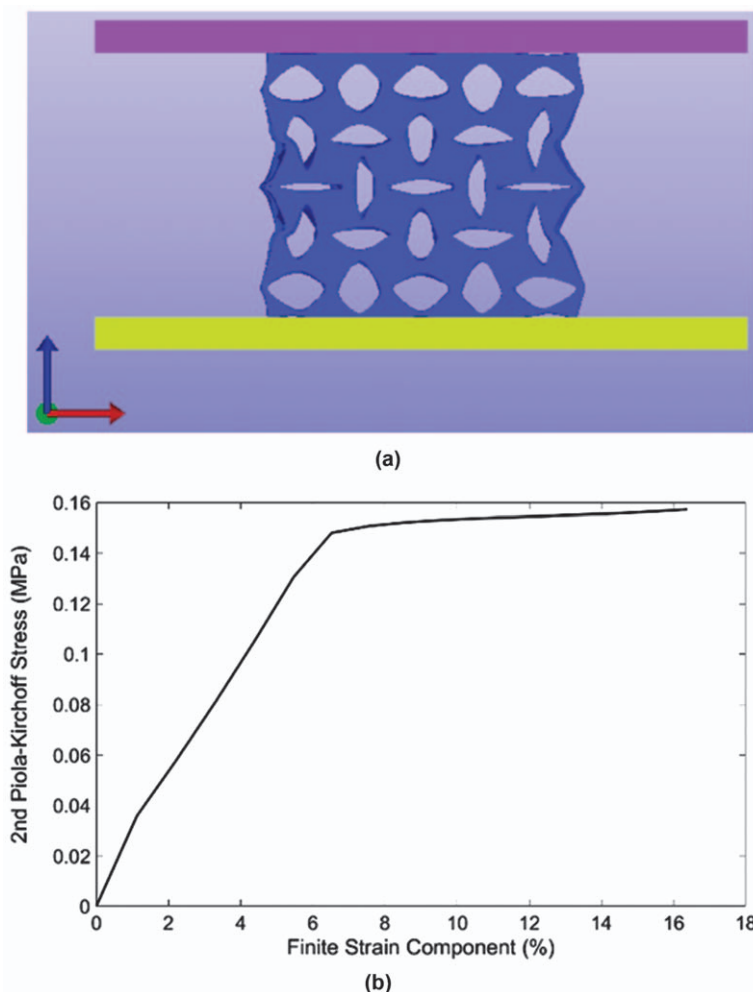


Figure 4.9 Example of (a) local scaffold architecture instability and (b) consequential loss of macroscopic stress-strain ellipticity as seen in global stress-strain curve with large displacement under small forces for 25% volume fraction P Schwarz architecture made from Neo-Hookean PGS material.

With regard to question 3, estimates of effective material coefficients for similar for linear elastic as well as the nonlinear elastic Neo-Hookean materials using both classic and mixed boundary homogenization methods. Furthermore, the classic Voight bounds provided upper bounds on both linear elastic and nonlinear elastic Neo-Hookean material effective coefficients. Although this result needs to be significantly expanded for other nonlinear scaffold materials, it provides an advantage for scaffold design using nonlinear elastomeric biomaterials. Nonlinear contact analyses using nonlinear scaffold materials are much more time consuming to perform

than linear elastic homogenization analyses with either classic or mixed boundary conditions. If the homogenization analyses can reliably estimate the percentage change in effective material coefficients for nonlinear materials, this will greatly enhance the number of scaffold architecture designs that can be screened for soft tissue engineering.

The ultimate application of these analyses and the answer to these questions is of course to design scaffolds that can replicate soft tissue behavior as characterized by effective nonlinear constitutive models^{19–24} (Figures 4.7 and 4.8). Figures 4.7 and 4.8 demonstrate that estimating effective scaffold nonlinear properties in relation to nonlinear tissue properties is the first step in determining the scaffold architecture designs and biomaterials that may be best suited to regenerating a given soft tissue. Furthermore, a common design scaffold design hypothesis in functional tissue engineering^{1,2} is that tissue engineering scaffolds should replicate the mechanical behavior of the tissue to be regenerated. Until we can computationally design a variety of architectures, reliably estimate their linear and nonlinear mechanical and mass transport properties, and fabricate these scaffolds from a wide range of biomaterials, we will not be able to test the hypotheses implicit in functional tissue engineering. Specifically, unless we can controllably design and fabricate scaffolds with known effective properties and their relationship to native tissue properties, we cannot test functional tissue engineering hypotheses and develop rigorous tissue engineering scaffold design criteria. This is not solely an academic exercise, FDA approval and Good Manufacturing Practices (GMP) rests on Design Controls, the basis of which is defining rigorous design inputs (*i.e.* design requirements).²⁸

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CHAPTER 5

Shear Thinning Hydrogel-based 3D Tissue Modelling

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5.1 Hydrogels: A Versatile Bioink Platform for Tissue Engineering

5.1.1 Advantages and Challenges of Gel-phase Bioinks

The first and most widely used bioinks for 3D bioprinting consisted of sol-phase inks that were extruded, blotted, or laser-deposited to create tissue constructs.^{1–3} A sol-phase ink is typically crosslinked only once using ultraviolet (UV) light immediately following deposition of each droplet, filament, or layer. Sol-phase inks were initially attractive because they are inherently printable and have a relatively straightforward crosslinking mechanism to form 3D constructs. While sol-phase inks are good for creating self-supporting scaffolds and can support cellular functions when cells are seeded onto them, sol-phase inks are not conducive for the printing of scaffolds with embedded cells (Figure 5.1). In a sol-phase ink, cells will settle to the bottom of the ink cartridge over time, causing inhomogeneity in the cell distribution and leading to clogged nozzles.^{4,5} There are also concerns that the photoinitiators used for UV curing of sol-phase inks can be cytotoxic in the cartridge over the long incubation times required for printing of complex 3D structures.⁶ Second, cells can be damaged during the printing process, as the fluid flow profile during liquid extrusion can rupture

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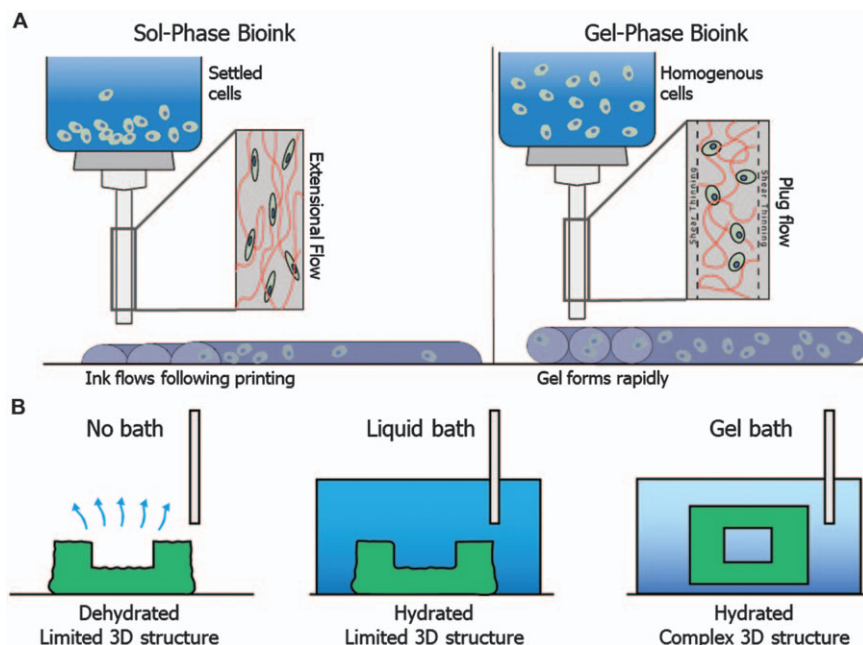


Figure 5.1 Methods of extrusion-based 3D bioprinting. (A) Gel-phase bioinks have many advantages over sol-phase bioinks. Ink gelation inside of the cartridge prevents cell sedimentation, and prevents cell damage during extrusion by undergoing “plug flow”. Immediately following printing, gel-phase bioinks quickly return to their gel-state, reducing the effects of ink flow on print fidelity. A homogenous cell suspension in the cartridge ensures even cell distribution inside the printed construct. (B) 3D bioprinting constructs without a bath can lead to dehydration of the construct and limits their 3D structure, while printing into a liquid bath will only prevent dehydration. Printing into a gel-like support bath prevents dehydration of the construct while enabling complex 3D structures that are not possible in a liquid print bath.

the cell membrane. Print fidelity also can be an issue, as the ink needs time to cure after it leaves the nozzle, which can cause loss of print resolution. Finally, UV crosslinking of sol-phase inks is typically performed in air, which can lead to potential dehydration and death of the cells. Many of these challenges for sol-phase inks are actively being addressed, but much work still needs to be done. In particular, sol-phase inks may not be well suited for long print times if cells are to be encapsulated within the ink, and long prints time will be necessary to achieve larger and more complex printed structures.

Sol-phase inks typically will have one crosslinking step that occurs immediately after printing. In contrast, most gel-phase inks have first- and second-stage crosslinking steps. The first-stage crosslinking is responsible for the weak, gel-like behavior of the ink in the cartridge. First-stage crosslinks are often dynamic in nature, *i.e.*, the bonds are reversible over time and

are constantly forming and reforming. During the printing process, the first crosslinks disassemble, enabling the gel to become fluid-like and extrudable. For the disassembly of the first crosslinks to occur, pressure is added to the gel in the cartridge, causing the gel to yield and flow. The pressure at which the gel begins to flow is defined as the yield point. After the gel-phase ink is printed, the first-stage crosslinks reassemble and second-stage crosslinking is used to further stabilize the hydrogel network. The combined effect of both first- and second-stage crosslinking is responsible for the final mechanical properties of the ink.

By combining a weak, first crosslinking step with a stronger, secondary crosslinking step, gel-phase inks are able to overcome many of the challenges associated with sol-phase inks. First, gel-phase inks prevent cell sedimentation during the printing process.⁷ Sedimentation is problematic during the long print times required for more complex and larger tissue engineering constructs. In sol-phase gels, gravity pulls cells to the bottom of the cartridge over the duration of the printing time. As cells sediment, they are no longer homogeneously dispersed in the ink, leading to differences in cell density in the final printed construct.^{4,5} This challenge can be partially mitigated by adding viscosity enhancers to the sol-phase ink, thereby slowing down cell sedimentation.⁸ In gel-phase bioinks, these problems are solved by adding weak, first-stage crosslinking to the ink in the cartridge. This gel-phase prevents cell sedimentation and maintains a homogeneous cell suspension. It has been suggested that gels with a yield point of ~ 5 mPa are sufficient to prevent cell sedimentation in the print cartridge.⁵ However, first-stage crosslinking that is too strong will lead to a larger yield point and hence require larger printing pressures, which could translate to poor printability of the ink. Preventing sedimentation of the cells in the ink while allowing for reasonable printing pressures should be a motivating factor when designing the first-stage crosslinking strategy of the gel-phase ink.

Second, some gel-phase inks are able to undergo “plug flow” to prevent cell damage during the printing process. Recent work has shown that mechanical forces experienced by cells during ejection can rupture the cell membrane and reduce cell viability.^{9,10} However, some gel-phase inks can undergo plug flow, which protects the cells from these damaging forces. In plug flow, the portion of the gel adjacent to the syringe walls is shear-thinned into a lower viscosity fluid. This lower viscosity fluid then serves as a lubricating layer that allows the rest of the gel and encapsulated cells to slip through the syringe as a relatively undeformed plug. Cells in the inner plug are not exposed to the mechanical forces exerted on the outer ink layer and remain viable throughout the printing process.⁸ In contrast, sol-phase inks, while often shear thinning, do not undergo plug flow, and their increased viscosity can lead to additional shear stress on the cell membrane. Thus, a key advantage of many gel-phase inks compared to sol-phase inks is that they can achieve higher cell viability during the printing process.

Next, gel-phase inks and their dynamic bonds are often printable into liquid media due to the rapid reassembly of the first-stage crosslinks. Printing

into a liquid medium prevents dehydration of biofabricated constructs and the associated cell death due to dehydration. While printing gel-phase inks, some of the dynamic crosslinks break, but large domains of undisturbed hydrogel remain.¹¹ Because few bonds are necessary to reconnect the intact hydrogel domains, the gel-phase ink returns to a solid nearly immediately after printing. Sol-based inks are liquids during extrusion, and their crosslinking is not immediate after ink deposition. Thus, the sol-phase ink is susceptible to flow before crosslinking, and this can lead to the dissolution of the ink if printed in a liquid medium. This problem can be solved by adding viscosity modifiers to the sol-phase ink or lightly crosslinking the ink before it leaves the nozzle by passing the solution through a glass capillary illuminated by UV light.¹² However, both of these modifications may decrease the printability of sol-phase bioinks. Additionally, printing into a liquid media bath is not conducive to rapid UV crosslinking of sol-phase inks, as the bath can scatter and attenuate the light.

In addition, many gel-phase inks are printable into gel-based baths that can enhance the final construct fidelity (Figure 5.2). These gel-based baths behave like solids that yield as the print head moves through them, leaving a channel for deposition of the gel-phase ink. With this yielding behavior, a gel-based bath acts as a Bingham plastic, only flowing after a yield stress is applied. Recently, three such gel-based baths have been reported, with their compositions and gelation mechanisms tuned to fit specific second-stage

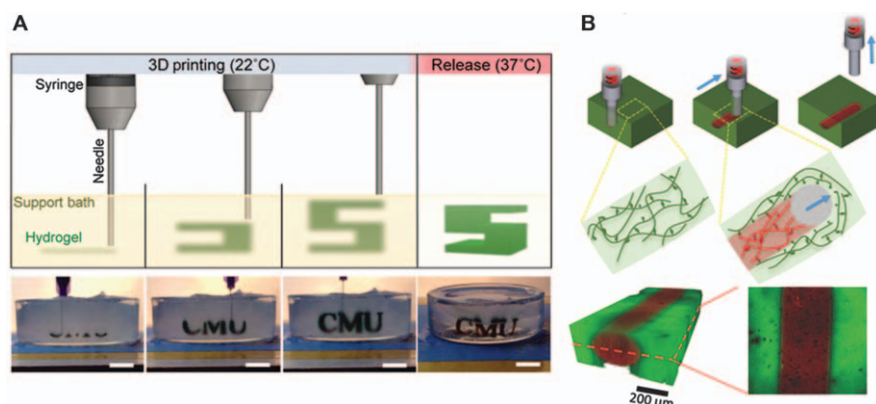


Figure 5.2 Bioprinting within a gel bath. (A) The FRESH printing process uses a gelatin slurry support bath to print free-form 3D structures at room temperature. The final print is released by melting the gelatin. (B) Gel-in-gel printing of cyclodextrin- and adamantane-modified HA hydrogels. The support gel yields when an extrusion head moves through it, and the gel-phase bioink is deposited to form a 3D pattern, thereby creating a composite structure of two gels.

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crosslinking mechanics.^{13–15} First, a support bath containing a gelatin slurry composed of micron-sized gel domains was shown to enable high print resolutions with inks using divalent cation crosslinking. The printed hydrogel stays in place until the gelatin slurry is melted at elevated temperatures to release the construct.¹³ This process has been named free-form reversible embedding of suspended hydrogels, or FRESH. While the FRESH printing process was designed to complement divalent cation secondary crosslinking, it is amenable to other secondary crosslinking schemes as well. Similarly, a “granular gel” was made of Carbopol, a poly(acrylic acid) polymer, to support 3D bioprinting.¹⁴ Again, micron-sized gels of the hydrophilic polymer formed a support bath that was designed for thermal first-stage crosslinking. After thermal crosslinking, the construct can be removed from the bath, and a second-stage crosslinking can occur. Taking a different approach, a solid guest–host gel bath was used to support the printing of a guest–host gel-phase bioink.¹⁵ Much like the gel-based slurries mentioned earlier, the solid gel bath was able to yield and shear thin during the printing process. Using this gel-in-gel technique, high print resolutions were demonstrated. For all three gel bath demonstrations, the high print resolution is due to the spatial confinement that the gel bath imposes on the printed bioink. In addition, another key advantage of gel-based bath printing is the ability to print free-form 3D structures that would not be possible without using a support gel. Finally, these gel-based baths are aqueous media, preventing dehydration of the construct.

5.1.2 Current Gel-phase Bioinks

While gel-phase inks have several advantages over more traditional sol-phase inks, their adoption for 3D bioprinting has been slow. However, ever more examples of gel-phase inks are being developed, and in this next section we highlight some current examples.

While alginate is a widely used material for sol-phase bioinks, alginate was made into a gel-phase ink by combining a peptide-modified alginate with an engineered protein.⁷ First-stage crosslinking in the ink occurs through molecular recognition between two peptide groups, one a proline-rich peptide and the other a computationally-derived WW binding domain. The proline-rich peptide is displayed on the alginate polymer, and seven copies of the WW domain are included on a recombinantly expressed protein backbone. In the cartridge, reversible first-stage crosslinking occurs through assembly of these two peptide domains, which allows the gel to shear thin and yield while printing. The gel-phase ink is printed into an aqueous medium that contains calcium to induce the second-stage crosslinking of the alginate backbone. After second-stage crosslinking, the printed constructs have a stiffness of 4 kPa. This gel-phase ink prevents cell sedimentation and cell membrane damage compared to alginate sol-phase ink. This ink was used to print and pattern multiple layers with different cell types in distinct spatial patterns.

Another recent bioink uses similar protein engineering approaches to create spider silk hydrogel inks.¹⁶ Spider silk hydrogels create first-stage crosslinks spontaneously at 37 °C by forming physical crosslinks between the β -sheet structures of the silk domains. Upon application of force, the hydrogel formed in the cartridge thins and reforms immediately after printing to form a 200 Pa gel that does not require secondary crosslinking. These spider silk scaffolds contained an arginine-glycine-aspartate (RGD) cell-binding ligand for cellular engagement and enhanced cellular function of the encapsulated cells. Cells encapsulated inside of the silk gels before and after printing were 70% viable.

As previously mentioned, guest–host hydrogels were designed for use as a gel-phase bioink and as a solid gel-support bath. Specifically, these materials exploited guest–host interactions between adamantane- and β -cyclodextrin-modified hyaluronic acid (HA).¹⁵ HA is a biocompatible extracellular matrix material that is known to be biochemically active and to support cell culture.¹⁷ Cyclodextrin is a host macrocycle with a hydrophobic core that can spontaneously assemble with the hydrophobic guest, adamantane.¹⁸ When adamantane-modified HA is mixed with a cyclodextrin-modified HA, a supramolecular hydrogel is formed with first-stage crosslinks due to guest–host bonds from the adamantane-cyclodextrin assembly. This guest–host ink allows for tuning of the final mechanical properties either by tuning the HA functionalization with the two guest–host moieties or HA polymer concentration. This bioink was also modified to enable UV crosslinking as a secondary mechanism to stiffen the construct after printing. By printing the gel-phase ink into a gel-phase bath of the same material, 3D structures were created that would not be possible without the use of a support gel.

It was recently demonstrated that 35 different hydrogel-ink formulations that contain a host of natural and synthetic materials, including gelatin, fibrin, collagen, and polyethylene glycol (PEG), could all be crosslinked using short, bifunctional PEG crosslinkers.¹⁹ The first-stage crosslinking was through succinimidyl valerate (SVA) reacting with free amines decorating the polymer backbones, while second-stage crosslinking was performed using UV crosslinks or common carbodiimide chemical crosslinking. As this gel-phase ink does not have reversible bonds, the degree of first-stage crosslinking is minimal, so that the material can still be printed despite having no dynamic bonds. The initial printed stiffness of the gels ranged from 1 to >150 Pa, and each of the 35 formulations was shown to be printable using extrusion-based techniques. While no cell studies were present, all of the chemistries used in this bioink library are cell compatible and could be used immediately for cell work. This large combinatorial approach to bioink development provides many bioink formulations that can be widely tuned and could be useful for designing tissue-specific hydrogel scaffolds.

Using a similar strategy, a bioink was developed using modified PEG to act as first- and second-stage crosslinkers with thiolated extracellular matrix (ECM) components.²⁰ The ECM components, bifunctional PEG-diacrylate, PEG-alkyne, and a photoinitiator were mixed together in the ink cartridge.

First, bifunctional PEG-diacrylate (PEG-DA) forms spontaneous first-stage crosslinks with thiol-modified HA and gelatin to form a weak, extrudable gel. After printing in the air, secondary crosslinking is accomplished using UV light to trigger a thiol-yne reaction between the PEG-alkyne and the sulfhydryls on the ECM components. This combination of first- and second-stage crosslinking results in printed inks with moduli from 100 to 20 000 Pa, which covers the stiffness range of most soft tissues. These gels supported the successful culture of cell spheroids, suggesting their potential future use as a versatile ink library for the creation of tissue constructs.

In summary, while gel-phase inks have many potential advantages compared to sol-phase inks, only a few examples of gel-phase inks have been published to date. A wide variety of possible first- and second-stage crosslinking mechanisms are available when designing new gel-phase bioinks. This versatility has already begun to result in the creation of libraries of inks with materials properties that can be modified and optimized for specific biomaterial applications. In the next section, we will discuss how these different bioink material properties may impact cell biology, including cell survival and cell phenotype.

5.2 Hydrogels as Tissue Engineering Scaffolds

Hydrogels have been widely used for tissue engineering applications because they can emulate many of the key matrix–cell interactions of native tissue.^{21,22} Cells are exquisitely sensitive to several different hydrogel properties. Thus, these hydrogel–cell interactions must be carefully considered when designing or selecting a gel-phase bioink for a specific biomedical application. In this section, we discuss key findings from the field of tissue engineering that highlights several hydrogel material properties that must be appropriately controlled in future bioink formulations.

5.2.1 Oxygen and Nutrient Transport

In any tissue engineering scaffold, proper nutrient and waste transport to and from the encapsulated cells must be ensured. For most hydrogel scaffolds, this transport is due to diffusion through the hydrogel polymer network. In the case of Fickian diffusion, the characteristic time required for a molecule to diffuse to the interior of the hydrogel is proportional to the diffusivity and inversely proportional to the square of the diffusional distance, $t \sim DL^{-1}$.²³ Changing either the diffusivity through the hydrogel or the distance a molecule must diffuse from a free surface can greatly alter the available oxygen and nutrient concentration within the interior of a scaffold. The diffusivity of molecules through a hydrogel varies depending on the size of the diffusing species relative to the hydrogel mesh size, which is dictated by the total polymer fraction and the density of polymer crosslinks.²⁴ For drug delivery from a bioink, diffusion may need to be limited to retain the cargo for longer release times. In contrast, for scaffolds with encapsulated cells, maximizing diffusion will increase nutrient and oxygen supply to

the cells. Thus, competing factors may be at play when selecting an optimal hydrogel formulation. However, the final size and shape of the printed construct may be even more important to consider. Cells more than a few millimeters away from a free hydrogel surface will not be able to survive, as oxygen and nutrient transport will be limited. There are many different bioprinting techniques currently being developed to create perfusable channels that allow medium to flow throughout a cell-laden construct.^{25,26} As these 3D constructs continue to increase in complexity, the amount of free hydrogel surface will also increase, thus enabling more efficient nutrient and oxygen transport and the fabrication of larger tissue constructs.

5.2.2 Incorporating Biochemical Signals

A well-designed hydrogel scaffold will promote cellular functions that match the desired tissue application. *In situ*, cells receive many types of biochemical signals from their surroundings that instruct their development and ultimate phenotype and function (Figure 5.3). For example, cells attach to and receive signals from the surrounding ECM through cell-adhesive peptide ligands that bind to cell-surface receptors. In addition, cells are also influenced by soluble growth factors and cytokines that may be specific to individual tissues.

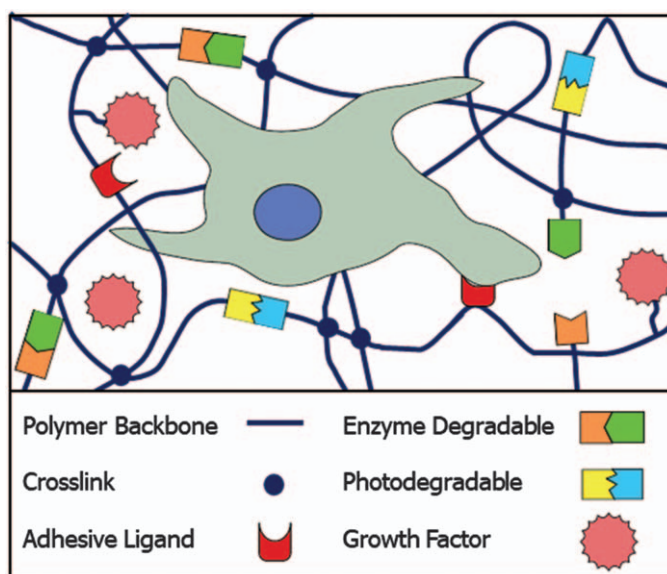


Figure 5.3 Possible design variables for new bioinks. Many aspects of the local cellular environment can be controlled through careful design of the gel-phase bioink hydrogel. Modifying the molecular weight of the polymer backbone between crosslinks will tune the hydrogel mechanical properties. Biofunctionality can be added by including soluble and tethered growth factors and adhesive ligands. Network degradation can be engineered into the bioink using photodegradable or enzyme degradable sites, or by hydrolysis of the polymer backbone.

While there are many examples of biochemical signals incorporated into scaffolds in the broader biomaterials community, these same signals have not been widely found in the maturing field of 3D bioprinting. Identifying and incorporating these biochemical factors into gel-phase bioinks will be an important step in customizing materials for tissue-specific 3D-printed constructs.

Biochemical factors in hydrogels can be presented in several different ways: designed into the polymer backbone itself, tethered to the polymer network, or added to the aqueous solvent of the hydrogel. Two common examples of materials with biochemical functionality found within the polymer backbone are collagen and HA. These naturally occurring ECM materials are widely used as tissue engineering scaffolds because they contain biochemical motifs that are recognized by cell-surface receptors.^{27,28} Thus, cells will adhere to these native ECM materials without any modification, making them attractive base materials for bioink design. Alternatively, the cell-adhesive peptide ligands found in ECM proteins can be tethered to nonadhesive scaffolds to promote cellular adhesion. For example, alginate, a polysaccharide found in brown algae, has been modified with the RGD cell-binding peptide ligand which is widely recognized by many cells.²⁹ While this RGD motif is the most commonly used cell-adhesive peptide ligand, a broad array of other cell-adhesive peptide motifs have been identified in the native ECM and could be incorporated into gel-phase inks.³⁰ Finally, soluble growth factors and cytokines, or their engineered mimics, could be delivered with the scaffold to further enhance bioactivity. Soluble factors have been included in hydrogels for tissue engineering in a number of different ways.³¹ These include simple encapsulation for passive release, placed in capsules for prolonged release, and tethered to the network for sustained presentation.³¹ While growth factors have been widely incorporated into hydrogel designs for tissue engineering, they have not yet been included in gel-phase bioinks.

5.2.3 Mechanical Properties

Gel-phase bioinks need to form mechanically robust hydrogel networks to retain their shape and to create larger tissue engineering constructs. The mechanics of the hydrogel matrix are also important to encapsulated cells. It is well established that cells can sense the mechanics of their matrix.^{32,33} This mechanosensing has been found to regulate several cell behaviors including cell spreading,³² migration,³⁴ proliferation,³⁵ gene expression³³ and differentiation.³² Gel stiffness is often tuned by changing the total polymer concentration or the density of crosslinks, which—as discussed earlier—will also alter nutrient and oxygen transport through the material. In general, the final stiffness of a gel-phase bioink should be designed to elicit the desired mechanobiological response from the encapsulated cells while enabling adequate transport. This optimal stiffness may or may not match the stiffness of the adult, native tissue.^{32,33} For most gel-phase inks, the final mechanical properties are primarily determined by the second-stage crosslinking, since first-stage crosslinking typically results in weak gels.

The mechanical response of a hydrogel matrix to cellular forces is not constant over time, and thus, the mechanics of a gel-phased ink cannot be accurately described by a single stiffness value. Due to the dynamic nature of most first-stage crosslinks, gel-phase bioinks are often viscoelastic, *i.e.* their mechanical response to an applied load is time-dependent and has characteristics of both a fluid and a solid. When a force is applied to a typical gel-phase bioink, it will first deform like an elastic solid, but over time will flow like a liquid as the dynamic crosslinks rearrange. Thus, as encapsulated cells adhere to the polymer scaffold and apply tension, the matrix will flow, and the tension will be dissipated through stress relaxation of the hydrogel. Recently, reports have demonstrated that cells also can respond to this time-dependent mechanical environment, but these effects are not yet fully understood.^{36,37} The extent of viscoelasticity in the final tissue engineered construct is a consequence of both first- and second-stage crosslinking. For example, permanent covalent second-stage crosslinking will reduce the viscous flow enabled by dynamic first-stage crosslinking in the final construct.

5.2.4 Degradability

Another important design criterion for gel-phase inks is the degradability of the matrix, *i.e.* hydrogel weakening and degradation and the weakening over time. Hydrogel degradation can be necessary for several reasons: cells often must degrade their matrix to spread and proliferate in chemically crosslinked hydrogels,^{38,39} matrix degradation can be required for important cell–cell contacts to form,^{40,41} hydrogel degradation can trigger the release of biochemical signals from depots,³⁹ and scaffold degradation is commonly a requirement for potential clinical applications.⁴² Just as there are many reasons that hydrogel degradation may be desired, there are equally as many ways to design a hydrogel to degrade using internal or external stimuli (Figure 5.3). Many ECM-based hydrogels are naturally degraded by cell-secreted protease enzymes.^{41,43} Synthetic hydrogels can be engineered to include amino acid sequences that are targets for proteases, thus making them degradable in response to cell-secreted enzymes.⁴⁴ Photoinitiated degradation is the cleavage of chemical bonds that are susceptible to specific wavelengths of light, enabling externally triggered degradation of the hydrogel scaffold.³⁹ This approach is an excellent means of modifying the matrix over time to study cell–matrix interactions. Hydrogels formed through physical crosslinks will erode over time, and the rate of this degradation can be tuned by altering the number and/or strength of the physical bonds. Finally, some hydrogels are designed to hydrolytically degrade, which is cleavage of chemical bonds by water.⁴⁵ This is particularly common in materials for clinical applications.

5.2.5 Hierarchical Structure

The local arrangement of polymers within a gel-phase ink into complex hierarchical structures can greatly alter the function of the encapsulated

cells. In natural tissue, organization of ECM components provides tissue-specific instructions for cells,⁴⁶ and incorporating these same signals into gel-phase bioinks may enable further control over cell responses. The most commonly observed hierarchical structure in hydrogel systems is the formation of fiber-like geometries that can guide cell migration and create anisotropic mechanical properties.^{34,47} At a macroscopic level, a fibrous hydrogel may have a stiffness that is comparable to a typical amorphous hydrogel, but on the length scale of an individual cell, the stiffness can vary greatly.³⁴ Different sized fibers can also affect how a cell adheres to and migrates within its surrounding matrix.⁴⁸ In addition, including nanofibrous components in a hydrogel can greatly enhance the mechanical stiffness and increase printability.⁴⁹ Microscale porosity can also be incorporated into hydrogel scaffold designs.^{50,51} The mesh size of most common, chemically crosslinked hydrogels is very small, only tens of nanometers, and thus cells cannot migrate freely through them. Including large, microscale pores can improve cell migration, both *in vitro* and *in vivo*.^{52,53} In addition to the hierarchical structures listed here, 3D printing may allow exploration of other structures that have not been previously possible.

5.3 Potential Crosslinking Mechanisms for Gel-phase Inks

As there are limited examples of gel-phase bioinks previously demonstrated, there are many opportunities to design new gel-phase inks to fit specific tissue engineering applications. In this section, we present several common hydrogel crosslinking strategies that may be useful for future 3D biofabrication studies. A complete gel-phase ink design will consider crosslinking strategies, scaffold materials, and final hydrogel properties equally to produce constructs that match the needs of a specific tissue engineering application.

First- and second-stage crosslinking have different roles when designing a gel-phase ink. As discussed earlier, the first-stage crosslinking is responsible for gelation inside of the print cartridge, preventing cell sedimentation, creating a plug flow during printing, and maintaining the gel structure after printing. First-stage crosslinking is generally accomplished through weak, reversible bonds that allow the bioink to flow. Second-stage crosslinking is used to create a more stable and durable gel after the printing has been completed. These bonds are typically stronger and more permanent bonds that do not allow for disassembly of the matrix. Many of the most common crosslinking strategies for hydrogels that have been used for years in tissue engineering can also be leveraged for use as bioink crosslinking mechanisms (Figure 5.4).

5.3.1 Guest–Host

Guest–host crosslinking is one type of supramolecular assembly where two or more molecules specifically interact to create a transient bond. Guest–host

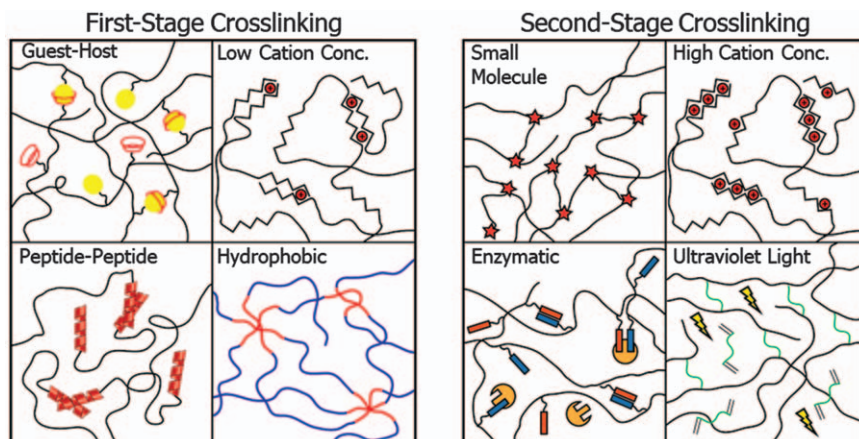


Figure 5.4 Potential first- and second-stage crosslinking methods. There are many possible ways to crosslink a gel-phase bioink using both first- and second-stage crosslinking. First-stage crosslinks are often dynamic physical bonds enabling reversible disassembly of the gel during extrusion. Guest-host systems, peptide-peptide assembly, electrostatic crosslinking using low divalent cation concentrations, and nonspecific hydrophobic interactions are all available first-stage crosslinking mechanisms. Second-stage crosslinks are typically permanent chemical bonds that enable increased stiffness and network durability after printing. Some common second-stage crosslinking mechanisms include use of small molecule covalent crosslinkers, enzymatic crosslinking, electrostatic crosslinking using high divalent cation concentrations, and use of ultraviolet light-induced photo crosslinking.

bonds assemble between a cavity-containing macrocycle and a separate molecular guest that fits within the macrocycle cavity to form a dynamic crosslink.⁵⁴ Guest-host molecules can be conjugated onto a hydrogel backbone to induce assembly of a polymeric network. One key advantage of guest-host systems is the specificity of the guest molecule for a particular host. The cavity of the macrocycle only accepts specific guest molecules based on the size, charge, and hydrophilicity of the cavity.⁵⁵ This specificity enables assembly of guest-host hydrogels only in the presence of the complementary guest molecule, allowing for multiple orthogonal guest-host interactions to be designed into a single hydrogel network.⁵⁶ Guest-host hydrogels commonly have rapid gelation kinetics, enabling quick recovery of gel-like behavior after shear thinning.¹⁸

The most common host macrocycles are cyclodextrins, a family of oligosaccharides with 6, 7, or 8 sugar units per ring.⁵⁷ The inner cavity of cyclodextrins are relatively hydrophobic compared to the hydrophilic exterior and can host many different hydrophobic molecules, the most common of which are adamantanes.⁵⁸ These guest-host molecules have already been successfully translated into a gel-phase bioink design¹⁵ and have been used extensively in the tissue engineering community for assembly of hydrogels and drug delivery.^{59,60} Another highly studied guest-host system is cucurbituril

macrocycles containing between 5 and 14 repeat units with greatly varying cavity sizes.⁶¹ These synthetic host molecules have highly specific and tight binding partners to their guest molecules, which is dependent on the host ring size.^{62,63} Some cucurbituril hosts can accommodate two distinct guest molecules, simultaneously enhancing the guest–host stability.⁵⁶ While these guest–host molecules have not yet been used in bioink design, they can form injectable hydrogels that have proven useful for drug delivery.^{64,65}

5.3.2 Peptide–Peptide

Another type of supramolecular assembly for crosslinking hydrogels is peptide–peptide interactions. Protein self-assembly into hierarchical tissues are responsible for many of the complex structures found in the body and are well suited for first-stage crosslinking of bioinks. Peptide–peptide assembly commonly occurs through physical bonds (including hydrogen bonds and electrostatic interactions) between two or more peptide domains.⁶⁶ Collagen is the best known example of peptide assembly in the body. Collagen, and its derivative gelatin, can self-assemble into triple helix bundles that may further assemble into fibers.⁶⁶

Engineered peptides and proteins are also attractive materials made from amino acids for creating tissue engineering scaffolds because of their inherent cytocompatibility and exact amino acid sequence control, which can include a large host of structural and bioactive peptide domains.⁶⁷ As highlighted in Section 1, two engineered peptide/protein bioinks have been reported to date. First, β -sheet interactions have been used to create silk-like bioinks,¹⁶ and interactions between proline-rich peptides and WW domains were used to create gel-phase bioinks.⁷ While these two examples represent the current state of bioink development, there are many possible protein/peptide crosslinks that have been successfully demonstrated in other biomedical applications and could be used for future bioink designs. For example, many different varieties of collagen-mimetic peptides have been designed that can self-assemble into materials.⁶⁸ Another form of helical self-assembly can be found in leucine zippers which have also been exploited in the design of several hydrogels.^{69,70} Other commonly used domains for engineered peptide/protein hydrogels include cartilage oligomeric matrix protein (COMP),⁷¹ α -helical peptides,⁷² β -hairpin hydrogel network,¹¹ and a “Dock and Lock” binding pair formed between domains from cAMP-dependent kinase A and A-kinase anchoring protein.⁷³ While this is not an exhaustive list of the available protein–protein interactions that can be used to create hydrogel materials, it is sufficient to demonstrate the wide breadth of peptide domains that are available for creating new bioinks.

5.3.3 Nonspecific Hydrophobic Interactions

Not all first-stage crosslinking mechanisms demand specific and complementary molecular interactions. Many hydrogel scaffolds use nonspecific aggregation of hydrophobic domains to form weak physical gels that match

the characteristics of a first-stage crosslink.^{74,75} In gels with hydrophobic interactions, hydration of the hydrophobic domains is thermodynamically unfavorable due to the formation of a well-ordered solvation layer, decreasing the total entropy of the system, leading to a positive free energy of solvation.⁷⁶ This will drive hydrophobic domains to associate together, excluding water and forming hydrophobic aggregates that physically crosslink the polymer chains. By designing polymers with hydrophobic regions, a successful bioink can be created.

Some of the most common hydrophobic crosslinking gels in tissue engineering are block copolymers, created using blocks of distinct polymer structure, with some blocks being hydrophobic and some blocks being hydrophilic. The most common block copolymers for tissue engineering applications are poly(ethylene oxide)-poly(lactic acid) (PEG-PLA) and poly(ethylene oxide)-poly(oxypropylene)-poly(ethylene oxide) (poloxamers), known by the trade name Pluronic. PLA is an attractive synthetic polymer for use in tissue engineering constructs owing to its biodegradability, but it requires hydrophilic PEG blocks to adequately solvate the hydrogel network.^{77,78} These materials were first investigated for use in drug delivery but have potential as candidate materials for bioprinting. Pluronics have been used as sacrificial inks in tissue engineering scaffolds due to their thermally reversible hydrophobic aggregation.^{25,75,79} Pluronic thermal responsiveness can be tuned by changing the molecular weight of each polymer component, and can be carefully chosen to match a specific printing application.

Another frequently used strategy to introduce hydrophobic crosslinking into a hydrogel network is tethering of poly(*N*-isopropylacrylamide) (PNIPAM) to otherwise hydrophilic polymer backbones. PNIPAM undergoes thermal aggregation at temperatures above 32 °C and has been tethered to peptide-peptide crosslinked materials,^{80,81} HA,⁸² and gelatin⁸³ networks, among others. By tethering PNIPAM to the backbone, the mechanical properties of the resulting material become thermo-responsive. An increase in temperature above the PNIPAM aggregation temperature will typically stiffen the matrix, allowing for modulation of the mechanical properties.

Recently, hydrophobic effects have been incorporated into shear thinning polymer-nanoparticle (PNP) hydrogels.^{84,85} Here, hydrophobic nanoparticles are placed in solution with polymers containing both hydrophilic and hydrophobic blocks. The surface of the particles is decorated by the hydrophobic chains from many different polymers, forming a gel network. These PNP materials are inexpensive and injectable, making them excellent candidates for creating 3D biofabricated constructs.

5.3.4 Calcium Crosslinking

Another type of physical crosslinking that can be considered for creating new bioinks is electrostatic interactions to create dynamic hydrogel networks. Most commonly found with divalent cation crosslinking, electrostatic

crosslinking is capable of forming gels with a wide range of stiffness and viscoelastic behavior, making electrostatic crosslinking a candidate for both first- and second-stage crosslinking.^{37,86}

Alginate, a derivative of brown seaweed, is the most widely used electrostatically crosslinked biomaterial. Alginate is a block copolymer of guluronic and mannuronic acid, with large blocks of guluronic acid primarily responsible for complexing with divalent cations, usually calcium, to create crosslinks between chains. Alginate has been widely used as a scaffold in tissue engineering due to its availability and cytocompatibility,^{53,86,87} but, due to its lack of inherent biochemical activity, does require modification with cell-adhesive ligands to mediate cellular interactions.²⁹ Owing to its ease of use, alginate has already been incorporated into several bioink designs.^{7,49,88}

5.3.5 Enzymatic Crosslinking

Outside of electrostatic crosslinking, the best candidates for secondary crosslinking in a bioink create covalent bonds between polymer chains to form a static hydrogel network following bioink deposition. One method for forming these covalent bonds is the use of peptide substrates and enzymatic crosslinking.⁸⁹ Enzymatic crosslinking requires two substrates that are recognized by the enzyme, and a covalent bond is formed between the substrates. The specificity of the interaction is enzyme dependent, as many of the enzymes used are derived from various bacterial and mammalian hosts. Multiple possible substrates and enzymes could be chosen for a single biomaterial to create orthogonal crosslinking strategies with distinct crosslinking events.⁹⁰ Enzymatic crosslinking is often attractive over other forms of chemical crosslinking because no harmful solvents are necessary and no harmful products are produced, although enzymes are typically more expensive than synthetic crosslinkers.

Transglutaminase was one of the first enzymes used to crosslink hydrogel structures.^{91,92} It is relatively nonspecific and will crosslink glutamine residues to lysine residues. The kinetics of bond formation can be tuned by altering the sequence of flanking amino acids.⁹¹ Lysyl oxidase is another enzyme that has been used to crosslink hydrogel networks.⁹³ With lysyl oxidase, primary amines of lysine residues are oxidized to aldehydes, which will spontaneously crosslink with other primary amines in the hydrogel. Like transglutaminase, lysyl oxidase is an attractive enzymatic crosslinker due to its broad substrate specificity, but this limits its usefulness when designing orthogonal enzyme crosslinking strategies.

A more specific enzyme that has been used to create tissue engineering hydrogels are sortases, a class of enzymes derived from Gram-positive bacteria. Many different bacteria contain sortases with unique substrate specificity, meaning multiple sortases can be used orthogonally to crosslink a polymer.⁹⁴ Sortases have already been used to design orthogonal crosslinking strategies to effectively tune a tissue engineering construct.⁹⁰

Finally, recent reports have presented the use of a novel, autocatalytic protein, SpyCatcher, to form covalent bonds within hydrogels.^{95,96} The SpyTag-SpyCatcher system creates an isopeptide bond that is substrate specific without the use of additional enzymes and can be incorporated into recombinantly expressed proteins or appended to any hydrogel backbone to successfully encapsulate cells.

5.3.6 Small Molecule Linkers

Small molecules with bi- or multi-functionality for the formation of covalent bonds between multiple polymer chains have been studied extensively for use in hydrogel biomaterials. While many of these small molecules could be candidates for use in bioinks, there are factors that should be considered when choosing an appropriate crosslinker and crosslinking chemistry. First, the crosslinking should not create harmful byproducts that could damage encapsulated cells. Second, the crosslinking must crosslink the material on a timescale that is useful for the final printed construct. Finally, the crosslinking should be chosen so as to minimize cross-reactivity with encapsulated cells, known as bio-orthogonality.⁹⁷

Many types of copper-free “click” chemistries have been used successfully to encapsulate and culture cells.^{98–100} Bio-orthogonal click chemistries are highly selective, high yield reactions between chemical species that are not found in nature, with minimal side products, making them excellent candidates for second-stage crosslinking.⁹⁷ The choice of which click chemistry to use is largely based on reaction times, as the click crosslinking reaction may occur over the course of seconds to hours depending on the chemical species involved.⁹⁹ Another class of reactions that are commonly used to form cell-laden hydrogels are thiol-reactive Michael type additions. This chemistry is well suited for use in bioink design due to its relatively low cross-reactivity and tunable reaction kinetics.¹⁰¹ Here, strong electron donors, such as thiol groups, are added to electron acceptors, which can include maleimides,¹⁰² vinyl sulfones,^{41,44} and alkyl methacrylates.⁵⁹

5.3.7 UV Crosslinking

Some of the first sol-phase bioinks created were crosslinked using ultraviolet (UV) light, and this same strategy can be employed in gel-phase inks as a second-stage crosslinker. UV crosslinking has always been attractive for use in biofabrication due to its ease of use and rapid crosslinking times. Popular UV crosslinking materials include poly(ethylene) diacrylate (PEG-DA) and gelatin methacrylate (GelMa). More recently, thiol-norbornene materials have been reported with bio-orthogonal crosslinking reactions and very rapid gelation kinetics.^{103,104} Many of the UV curable sol-phase inks require the use of cytotoxic photoinitiators, including Irgacure and lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP), which can result in a 20% or more reduction in cell viability after only one hour of exposure.^{6,8,105} Thus, the use

of these photoinitiators may not be feasible with the long print times required for larger and more complex constructs. Additionally, as mentioned in Section 1, UV curing of sol-phase constructs in air can lead to significant cell death due to dehydration.⁸ Despite these limitations, second-stage photocrosslinking has been successfully incorporated into a gel-phase bioink.^{15,19,20} To overcome potential cytotoxicity challenges in the future, the photoinitiators could be added only to the support bath in the final print medium, thus minimizing exposure.

5.4 Complex Architectures Using Hydrogel Inks

Gel-phase bioinks are suitable for creating many different 3D printed structures including complex architectures that will be required for advanced tissue engineering constructs. Mimicking real tissue architecture will require many advanced bioprinting techniques that must be considered when choosing or designing a new gel-phase bioink. Possible complexity that can be included in an advanced construct is the encapsulation of multiple cell types, patterning of several different materials and biochemical factors to influence local cellular function, or use of sacrificial inks to create voids within larger constructs. 3D bioprinting using gel-phase inks is uniquely suited to handle all of these challenges in the future due to its flexibility and scalability.

Natural tissues are not composed of one cell type, but rather many cell types cooperating to generate the final tissue function. Most reports of bioinks to date have only included a single cell type, however proof-of-concept studies have demonstrated the ability to deposit multiple cell types inside of distinct bioinks for spatial patterning of cells (Figure 5.5).^{7,106,107} In a complimentary approach, a heterogeneous cell mixture can be printed within one bioink.¹⁰⁸ When delivering a homogeneous coculture of human nasal chondrocytes (hNCs) alongside human bone marrow-derived stem cells (hBMSCs) to create a cartilage analogue, significantly greater cartilage synthesis was observed after 60 days in a subcutaneous mouse model when compared to a chondrocyte ink alone.¹⁰⁸ Similarly, two separate bioinks were used to print MG63s, a human osteoblast-like cell line, and hNCs to recreate the spatial patterning of osteochondral tissue, *i.e.* tissue at the interface between bone and cartilage.¹⁰⁷ While both of these studies investigated the use of multiple cell types, much work remains to be done to optimize the printing process for different cell types. In particular, different cell types may have different medium requirements, may have different sensitivities to specific crosslinking strategies, and may have different responses to the material properties of the bioink.

Just as a native tissue is not cellularly homogeneous, its physical structure and arrangement of ECM proteins are characterized by inhomogeneity, including anisotropy, hierarchical structures, and gradients of physical and biochemical features. For this reason, creating spatial patterns of materials or biochemical factors may be beneficial for the future of 3D bioprinting to recreate the functionality of native tissue. One way of incorporating material inhomogeneity into a 3D printed construct is to use multi-ink extrusion

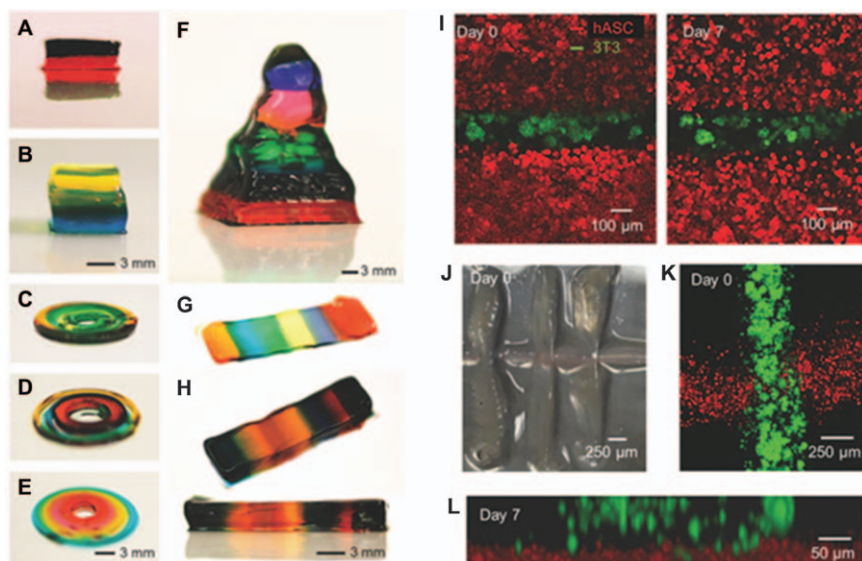


Figure 5.5 Patterning materials and cells in 3D bioprinting. Multiple bioinks can be combined to create 3D constructs with spatially distinct material properties, biochemical activity or cellular function. (A, B) Two and three materials used to print cubes. (C–E) Two, three, and four materials used to print transversely varied discs. (F) A printed pyramid containing seven different material layers. (G, H) Three and ten layers of continuously extruded blocks containing seven different inks. (I) Patterned human adipose-derived stem cells (hASCs) (red) and NIH 3T3 fibroblasts (green) maintain their spatial patterning over seven days. (J, K) Photograph and fluorescent image of perpendicular printed lines of materials containing hASCs and 3T3 cells. (L) Fluorescent cross-section of perpendicular printed lines containing hASCs and 3T3s after 7 days of culture showing spatial separation of distinct cellular inks. (A)–(H) adapted with permission from ref. 110, Copyright 2016 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim. (I)–(L) adapted from ref. 7 with permission from John Wiley and Sons, Copyright 2016 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

bioprinting, where multiple inks can be printed simultaneously into one printed construct, each with a distinct mechanical or biological function. This can be done by using separate nozzles for each bioink,¹⁰⁹ or using continuous multimaterial extrusion (Figure 5.5).¹¹⁰ While it has yet to be demonstrated, materials with varying biochemical factors could potentially be used to fabricate 3D printed constructs with spatial control over the cellular response. Finally, patterning of cell layers with varying cell density may further broaden the design space for creating 3D printed constructs. In one report, a computational model informed optimal cell densities for proper regeneration of biofabricated bone tissue.¹¹¹ After fabrication, the authors report that the spatially patterned cell densities were still present after 3 days, however potential functional differences in regenerative capacity were not assessed.

Another advanced fabrication technique that can be achieved with gel-phase inks is the use of sacrificial hydrogels to pattern 3D voids within larger constructs. To date, this has been explored primarily as a means to create artificial vascular channels.¹¹² These printed vascular channels can be made from many different materials, each with their own deposition and removal techniques (Figure 5.6).^{25,113} In one example, a carbohydrate glass was

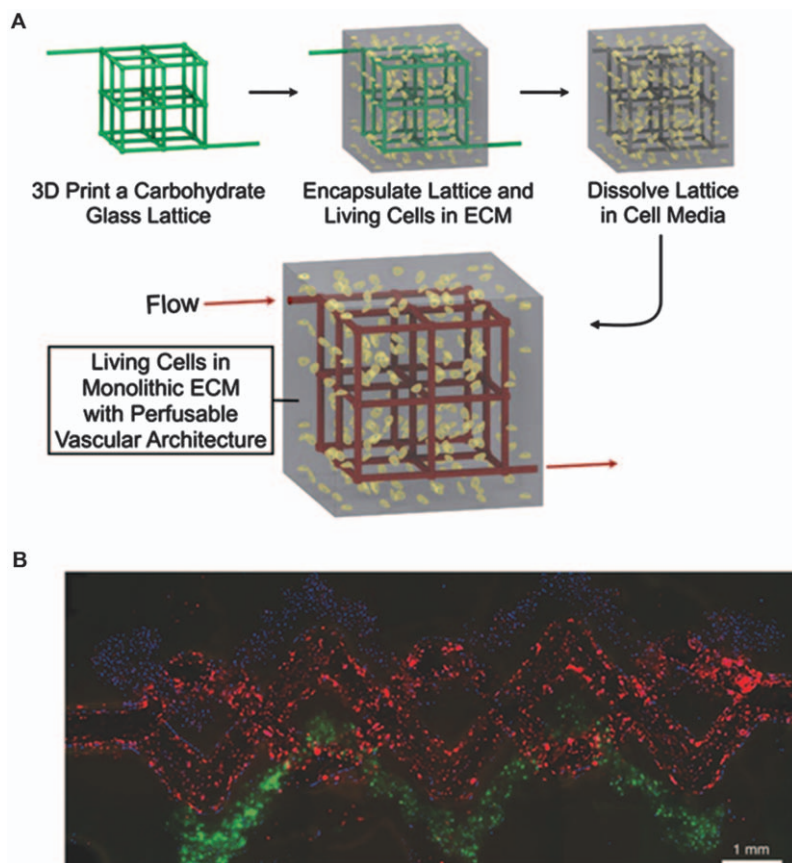


Figure 5.6 Two examples of sacrificial inks used to fabricate networks in “vasculature” constructs. (A) A carbohydrate glass is used to print a vascular network that is encapsulated within a second material containing cells. The carbohydrate glass is then removed through dissolution of the sugar molecules into the aqueous cell culture medium, leaving a hollow structure that allows for perfusion of the cast construct. (B) A Pluronic-F127 vasculature network is printed and then encapsulated within a second material hydrogel. Cooling the entire construct liquefies the Pluronic for removal to leave a hollow structure. Perfusion of endothelial cells repopulates the open channels.

(A) adapted from ref. 113 with permission from Springer Nature, Copyright 2012. (B) adapted from ref. 25 with permission from John Wiley and Sons, Copyright 2014 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

printed into a 3D network and then surrounded with a hydrogel, causing the sugar-glass to dissolve and leaving behind perfusable channels. In a second example, a Pluronic ink network was surrounded by a hydrogel, and cooled to liquefy the Pluronic for removal. In both cases, the perfusable networks were then filled with endothelial cells to emulate vasculature. Also worth consideration is the introduction of vascular channels within a printed line using coaxial needles to maintain an open network at the core of a 3D printed filament.¹¹⁴ In one demonstration of this strategy, alginate was extruded from the outer sheath of the coaxial needle, while a calcium solution was introduced through the core to immediately crosslink the alginate and maintain a hollow vascular space inside of the filament. While the use of sacrificial inks has been limited to the printing of vascular-like networks to date, in the future this technique could be combined with gel-support bath printing to achieve a wide array of complex 3D architectures with open void spaces.

5.5 Closing Remarks

While gel-phase bioinks with first- and second- stage crosslinking are quite promising and hold many advantages over sol-phase bioinks, there have been limited examples of such bioinks reported to date. When designing new gel-phase bioinks, careful consideration should be given to how cells may respond to the ink's material properties, the choice of first- and second-stage crosslinking mechanisms, and the ability of the ink to be used to create complex architectures. Each of these factors must work together to create a successful biofabricated construct. In the future of complex 3D biofabricated constructs, gel-phase bioprinting will play an important role towards creating biomimetic tissue structures.

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CHAPTER 6

Polymers in Biofabrication and 3D Tissue Modelling

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6.1 Sources of Polymer-based Biomaterials in Biofabrication and 3D Tissue Modelling

Polymers used in biofabrication and 3D tissue modelling can be sourced from nature, or synthesised *de novo*. Generally speaking, naturally-derived polymers exhibit intrinsic biofunctionality, which can range from cell-adhesion sites to enzymatic cleavage sites and signalling moieties. In principle, such functionality can be built into synthetic polymers as well. This has been performed to some extent, most notably the inclusion in hydrogels of the cell-adhesive arginine-glycine-aspartate (RGD) peptide sequence,¹ matrix metalloproteinase (MMP)-sensitive crosslinks² and network-bound vascular endothelial growth factor (VEGF) that can stimulate angiogenesis.³ However, the complexity of the native extracellular matrix (ECM) bearing a multitude of such functionalities has not yet been reproduced in a synthetic biomaterial. This is why naturally-derived polymers, including ECM derivatives, are still the most widely used in biofabrication and 3D tissue modelling. This is despite their typical disadvantages which include batch-to-batch variation, lack of tailorability and the risk of transmitting disease or

endotoxins. In this section we highlight some of the most widely used polymers in this research field, with a focus on hydrogel-forming polymers that can be used as bioinks.

6.1.1 Naturally-derived Polymers

6.1.1.1 Proteins

Collagen is the most abundant protein in the body. There are more than 15 types of collagen, but the vast majority of the collagen in the human body is either type I, II or III. It can be solubilised without loss of its natural conformation in acidic conditions and stored cold. When neutralised, there is a possibility to mix in cells and bioprint the collagen before it is fully solidified.^{4,5} Collagen gels generally are suitable environments for cells to proliferate and produce matrix. However, their slow gelation and weak mechanical properties often necessitate the use of auxiliary materials to fabricate tissue models.

Gelatine is the partly denatured form of collagen, mainly type I. It is prepared by boiling slaughterhouse waste including bones, skin and tendon in either acidic or basic conditions, rendering gelatine type A or type B respectively. Its inherent biofunctionality, wide availability, ease of processing and thermoresponsiveness make it a popular material for biofabrication. Gelatine naturally exhibits cell-adhesive peptide sequences, including RGD, and is amenable to cell-mediated degradation through MMPs. For this reason, it has also been used in bioinks as a complementary material to lend biofunctionality to bio-inert materials such as alginate⁶ and hyaluronic acid.⁷ A plethora of modifications have been performed to enable irreversible crosslinking of gelatine using different initiating methods (including light, heat, redox and enzymes), of which the combination of gelatine-methacryloyl (gelMA) with 2-hydroxy-4'-2-hydroxyethoxy-2-methylpropio-phenone (Irgacure[®] 2959) is by far the most used.⁸ Gelatine on its own can be 3D printed by extrusion methods with good shape fidelity at the appropriate temperature where it is partly physically crosslinked; solid enough to retain its shape after printing but not too solid to be extruded into smooth filaments.⁹⁻¹¹ GelMA has been printed on its own as well in this way, requiring a very strict control over temperature along the cartridge and nozzle.¹² However, more often gelatine is used in conjunction with other components to improve rheology. Gelatine and its derivatives have been used with different biofabrication techniques including extrusion bioprinting,⁸ inkjet printing¹³ and stereolithography.¹⁴

Fibrin is the tough fibrous protein responsible for blood clotting, and it is formed when its soluble precursor fibrinogen is exposed to the enzyme thrombin. This two-component system is exploited in the clinic in the form of a tissue glue for controlling bleeding (TISSEEL[®]) and is used in tissue engineering research as a cell-encapsulating hydrogel. It forms a highly permissive substrate that allows cells to proliferate, which has been used in

the biofabrication of various 3D tissue models.^{15,16} On their own fibrin gels are mechanically weak and fast-degrading (days to weeks) making them a poor construction material, which is why they are often used in conjunction with a scaffolding material, or blended with other gel components,¹⁷ or printed into a suspension bath (see Section 6.3.3.2).¹⁸

Silk is a proteinaceous material commonly obtained from the silkworm *Bombyx mori*, which lends itself well as a cell culture substrate due to high strength and flexibility, cell adhesiveness, low immunogenicity and appropriate degradation profiles. As with other proteins and decellularised extracellular matrices (dECM) (see Section 6.1.1.3) that were first used to prepare scaffolds for subsequent cell seeding, more recently silk has been processed into hydrogel form to allow cell encapsulation and bioprinting.^{19–21}

Matrigel[®] is a protein mixture secreted by Engelbreth–Holm–Swarm (EHS) mouse sarcoma cells, which has been used as a 3D substrate to culture cells and particularly organoids.²² The necessity for cooling to <4 °C to prevent premature gelation, the weak resulting gel and poor reproducibility in behaviour have limited its use in bioprinting, although some successful attempts have been reported.^{23,24} Nevertheless, Matrigel[®] is superior to most other gels in stimulating complex biological processes such as organoid formation. This superiority is directly linked to its poorly-defined and complex composition, including proteins such as laminin, entactin, collagen, as well as heparan sulphate proteoglycans and a cocktail of growth factors of unknown composition and concentration. This underlines why naturally-derived polymers are still the most heavily used class of materials in biofabrication and 3D tissue modelling: the better biological performance outweighs the inherent shortcomings of limited reproducibility and tailorability.

6.1.1.2 Polysaccharides

Polysaccharides are fairly bio-inert water-soluble polymers that can be obtained at high purity and at high molecular weights, resulting in high viscosities appropriate for the bioprinting process. Perhaps the most popular is alginate, which is a linear copolymer of guluronic and mannuronic acid extracted from algae (hence the name alginate) or seaweed. The ratio of the two monomers can vary, and this influences its properties—gelation behaviour in particular. Alginate is anionic and soluble in the presence of monovalent counter ions (e.g., Na⁺) but crosslinks to form a gel in the presence of multivalent ions such as Ca²⁺. As reviewed by Axpe *et al.*²⁵ it has been widely used in bioprinting either by itself (as inert matrix), in combination with other components such as gelatine, or in modified forms to promote cell attachment (e.g., through RGD functionalisation) or to augment degradation.

Different from the ionic crosslinking mechanism in alginate, is the gelation of thermoresponsive polysaccharides. These will solidify when heated above their lower critical solution temperature (LCST), or inversely, cooled

below their upper critical solution temperature (UCST). Examples of such polymers which have been employed as bioinks are seaweed-derived agarose (USCT),²⁶ plant-derived and subsequently alkoxyated celluloses (LCST),^{26–28} bacterially produced gellan gum (UCST in combination with ionic cross-linking),^{29,30} and crustacean-derived chitosan (UCST in combination with pH-dependency).³¹ Dextran, a polymer built up of solely glucose units, neither forms physical nor chemical gels without modification. However, it has been used in conjunction with other materials, including synthetic polymers (see Section 6.1.2) and gelatine, after functionalisation of the dextran with aldehyde groups that can crosslink with amines present on the gelatine chains.³²

Glycosaminoglycans (GAGs) are a sub-category of polysaccharides built up of a repeating disaccharide unit consisting of an amino sugar and a uronic sugar or galactose. Perhaps the most well-known GAG is hyaluronic acid (HA) or hyaluronan, which is an important ECM component of cartilage, tumours and other tissues, as well as of synovial fluid found in articulating joints. It has been employed in bioprinting research, either in combination with other components and/or after chemical modification to enable crosslinking, as HA does not have intrinsic gelation capability. It has been shown to improve the rheology for bioprinting of gelMA³³ and of methacrylated dextran.³⁴ Although methacrylated HA (HAMA or MeHA) has been printed on its own,³⁵ improved rheology and printability have been obtained through the addition of moieties that initiate reversible physical gelation through guest–host interactions, providing short-term stability to printed constructs prior to UV-initiated crosslinking of the methacrylate groups.³⁶ A similar approach was followed by Müller *et al.* who also used HAMA, but combined it with poly(*N*-isopropylacrylamide) grafted HA to obtain a thermoresponsive physical gel giving short-term shape stability.³⁷ Besides HA, a different GAG found in cartilage, chondroitin sulphate, has been used for cartilage tissue engineering^{38,39} and 3D bioprinting as well.⁴⁰

6.1.1.3 Decellularised Extracellular Matrix

Although dECM is a composite rather than a single polymer, it has played an important role in tissue engineering in general and is becoming increasingly important in biofabrication as well. The main challenges in the transformation of ECM into a useable bioink are: (1) the complete removal of cellular components and (2) processing it into a printable material bearing the right rheological properties, whilst (3) attempting to retain as much as possible of the intrinsic cues from the native ECM. A combination of procedures removes almost all of the cells and their remnants, however, most of the structural proteins are retained, in their native conformation.⁴¹ GAGs are typically partly lost, whilst the reduction of matrix-bound signalling molecules is not easily quantified. Although the decellularisation of ECM was firstly performed to prepare solid tissue engineering scaffolds, the conversion into hydrogels that allow cell encapsulation has become increasingly

popular, with a dECM hydrogel being in phase 1 clinical trial at the time of writing.⁴² This opens up the possibility of using dECM for biofabrication, which is still in its infancy but receiving particular interest.⁴³ The most prominent research in this area has been in the group of Dong Woo Cho at Postech in Korea, where they have been able to generate bioinks from adipose, cartilage and heart tissue, to re-engineer those same tissues using bioprinting techniques.⁴⁴ Another striking example is from ETH Zürich, where Matti Kesti *et al.* used a clinically approved decellularised cartilage particle product with (also FDA-approved) gellan gum and alginate to bioprint large, strong and functional cartilaginous structures.⁴⁵

6.1.2 Synthetic Polymers

Perhaps the most widely used synthetic hydrogel is poloxamer 407, which has excellent rheological properties for extrusion printing. It has been used both unadorned⁴⁶ and in modified forms, to perform various functions (see Section 7). A detailed description of poloxamer 407 and its uses bioprinting is found in Section 6.2.2 (poloxamer 407 case study) and in Section 6.3.3.4 on rheology modifiers.⁴⁷

Another thermoresponsive synthetic polymer that has been the basis for the development of several bioinks is poly(*N*-(2-hydroxypropyl)methacrylamide lactate) or poly(*N*-(2-hydroxypropyl) methacrylamide) (PHPMA) in the group of Wim Hennink. It has been employed as an ABA triblock copolymer with poly(ethylene glycol) (PEG) as the B-block.⁴⁸ In an attempt to tailor the ink to cartilage bioprinting, it has been supplemented with HAMA,⁴⁹ methacrylated chondroitin sulphate,⁴⁰ or both,⁵⁰ to optimize cartilage-like tissue formation by embedded chondrocytes, and enhance printability. PHPMA has also been used in the same group to prepare block copolymers with PEG and poly(*N*-isopropylacrylamide) (PNIPAAm). Here, the PHPMA was functionalised with cysteine residues, enabling crosslinking with *N*-hydroxysuccinimide (NHS) functionalised PEG or HA through oxo-ester mediated native chemical ligation.⁵¹

Poly(ethylene glycol) (PEG) is an inert water-soluble polymer that is prevalent in tissue engineering research, and has been used for the formulation of bioinks as well. PEG solutions typically exhibit low viscosity, making them suitable for inkjet printing,⁵² *e.g.*, in combination with acrylated peptides to improve biofunctionality.⁵³ Acrylated PEG has been employed in stereolithography fabrication of cell-laden structures,⁵⁴ also including PEG-RGDS to provide cell attachment sites for improved cytocompatibility.⁵⁵ PEG has been fabricated using extrusion bioprinting followed by direct photo-initiated crosslinking,⁵⁶ but more often it is combined with other materials to obtain higher viscosities and/or partial gelation of the ink, such as with alginate.⁵⁷ Perhaps the most beneficial use of PEG in bioink development has been to tune the properties of gels mainly made up of other polymers, such as the controlled crosslinking of thiolated HA using PEG-diacrylate.⁵⁸ Another neat example of this type of use is the employment

of PEG crosslinkers to tailor the rheological properties of a range of multi-material bioinks through controlled, homogeneously distributed crosslinks.⁵⁹

Li *et al.* harnessed the hybridisation of complementary DNA strands to crosslink polypeptides into a hydrogel, using this as a two-component bioink for bioprinting with a micro valve printer.⁶⁰ Although the components are synthetic, the bioinspired ink combines favourable properties of both the polypeptide and DNA components, including responsiveness to proteases and nucleases, and being fully biodegradable. The same holds true for self-assembling peptides amphiphiles, which are (short) synthetic peptides that self-assemble through hydrophobic interactions into nanofibers and potentially hydrogels. These have been studied since the 1990s and proposed for cell encapsulation since 2000,⁶¹ however only recently their use as bioinks in bioprinting has been proposed.⁶²

First steps are being made to decipher cell–matrix interactions to the molecular level to enable the development of fully defined synthetic ‘designer matrices’ for the culture of specific cells and organoids.⁶³ Although this is definitely the way forward towards full control over cell–material interactions and elimination of the intrinsic disadvantages of naturally-derived polymers, the efforts associated with the development of designer matrices for every possible cell and tissue makes it likely that less well-defined natural polymers, as well as over-reductionist synthetic materials will remain widely used for biofabrication and 3D tissue modelling over decades to come.

6.2 Properties of Polymer-based Biomaterials in Biofabrication and 3D Tissue Modelling

This section describes some of the most important properties and behaviours of polymers in biofabrication processes, focussing on the influence of physical properties on printability and cell behaviour, regardless of the biochemical and biological properties of the polymers.

6.2.1 Rheology

The field of rheology studies the deformation and flow of soft matter. When subjected to shear stress (τ in Pa), fluid will flow at a shear rate $\dot{\gamma}$ (s^{-1}), which is a dimensionless velocity. The resistance to flow is characterised by the fluid viscosity $\eta = \frac{\tau}{\dot{\gamma}}$. This relationship is important in biofabrication, as too

high shear stresses can damage cells, whilst too low shear rates imply slow building speeds. The simplest rheological behaviour a fluid can exhibit is called Newtonian, where the viscosity is said to be shear rate-independent, meaning shear stress and shear rate scale linearly. However, most polymer melts and solutions are non-Newtonian. Common types of non-Newtonian

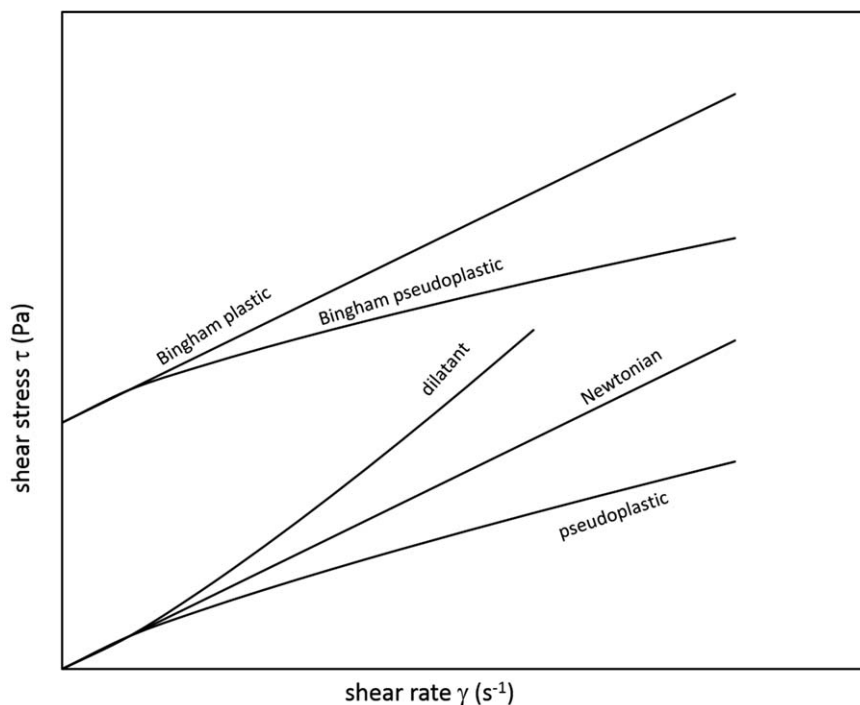


Figure 6.1 Different types of non-Newtonian behaviour; shear stress *vs.* shear rate.

behaviour are summarised in Figure 6.1. Other complex rheological behaviours not included in the figure are temperature-dependency, and thixotropy, where viscosity changes over time (even at constant shear rate). Rheological properties of bioinks are often quite complex, and of utmost importance for the ability to fabricate constructs with high shape fidelity.

6.2.1.1 Viscosity

For most biofabrication methods there is an upper and/or lower limit to the viscosity of materials that can be processed. Inkjet printing, for example, requires inks with a maximum viscosity of approximately 0.1 Pa s^{-1} .⁶⁴ This impedes the use of extracellular matrix components at high concentrations, as well as the building up of large 3D structures. A similar requirement is imposed for laser-induced forward transfer printing, in which inks with viscosities of up to 0.3 Pa s^{-1} are jetted from a carrier onto a substrate following a laser pulse that generates a pressure wave in the ink as a result of local evaporation.⁶⁵ Higher viscosities (up to several Pa s^{-1}) can be used in stereolithography 3D printing, which is based on the local solidification of a liquid resin by spatially controlled photo curing.⁶⁶

Extrusion-based bioprinting, in particular, relies heavily on viscosity, particularly on different viscosities during different stages of the printing

process. Ideally, during extrusion, the viscosity should be low to enable speedy fabrication without excessive shear stresses on the cells. However, it should still be high enough to counteract surface tension-driven droplet formation, in case the printing of filaments is desired (which is usually the case to obtain well-defined structures at high resolution). Directly after extrusion the viscosity should rapidly increase to avoid extruded filaments to flow and sag.

6.2.1.2 Shear-thinning

Shear-thinning behaviour refers to a decrease in viscosity with an increase in shear rate, as exhibited by pseudoplastic fluids (Figure 6.1). Nearly all polymer solutions are shear-thinning to some extent, at a sufficiently high concentration and molecular weight at least. This is due to a gradual change in conformation of the polymer chains from globular random coils to stretched linear chains as the shear rate is increased, leading to a decrease in entanglements and hence less resistance to flow.⁶⁷ This is advantageous for extrusion-based bioprinting, as a sharp decrease in viscosity at the high shear rates experienced in the nozzle allows higher flow rates to be achieved, whilst protecting the cells against excessive shear forces.⁶⁸ After deposition of the extruded filament, the shear stress and shear rate reduce to near-zero shear, resulting in a sharp increase in viscosity that slows down further undesired flow. Shear-thinning behaviour can be characterised on a rheometer by performing a shear rate sweep, in which torque (directly related to shear stress) is measured over a range of rotation speeds (directly related to shear rate), and fitted to the power-law model:⁶⁹

$$\tau = K\dot{\gamma}^n \quad (6.1)$$

in which K is the consistency index (Pa s^{-n}) and n the shear-thinning factor (dimensionless). For a Newtonian fluid $n = 1$; the smaller n , the stronger the fluid is shear-thinning. Dilatant fluids ($n > 1$) are less common, and have not received interest for biofabrication purposes.

6.2.1.3 Yield Stress

Although the shear-thinning behaviour is advantageous for extrusion-based bioprinting as described above, a pseudoplastic material will still flow due to gravity after deposition, causing a loss in shape fidelity of the printed construct. Contrarily, a Bingham pseudoplastic requires a minimum threshold stress to be deformed, or in other words has an infinite viscosity at zero shear rate. Many examples of such materials are encountered in everyday life (e.g., mayonnaise, hand cream, whipped cream) and they are often emulsions, colloids or physical gels with weak reversible crosslinks. When a bioink exhibits a yield stress that is higher than forces exerted by gravity, this aids in preventing collapse of printed shapes. At the same time, too high yield stress will inhibit the mixing of cells into the ink or even extrusion of

smooth filaments, so there is a maximum allowable range.³⁰ Yield stress has been exploited for bioprinting by partial chemical⁷ or physical cross-linking,¹⁰ by employing the ionic crosslinking of gellan gum with tailored ionic strength⁷⁰ or through electrostatic interactions between different bioink components, *e.g.*, negatively charged alginate with positively charged gelatine.^{71,72} For Bingham pseudoplastics, the power-law model is modified to include the yield stress τ_0 , according to the Herschel–Bulkley model:

$$\tau = \tau_0 + K\dot{\gamma}^n \quad (6.2)$$

6.2.2 Case Study: Poloxamer 407

Poloxamer 407 (also known as Kolliphor P407, Pluronic F127 and Lutrol F127) is a material that shows ideal rheological properties for bioprinting, when used as concentrated aqueous solutions. Poloxamer 407 is a triblock copolymer of poly(ethylene glycol) and poly(propylene glycol): PEG₉₁-PPG₅₆-PEG₉₁. At temperatures above its LCST (10–15 °C depending on concentration, minimum 15 wt%), the hydrophobic PPG segments aggregate and micelles are formed. When the concentration exceeds the critical aggregation concentration (CAC), the PEG coronas overlap and entangle, forming a physical gel with high but transient viscosity.⁷³ The resulting gel displays a yield stress of several hundred Pa, which is enough to prevent sagging of printed filaments but is not too high to be overcome by the pressure in the syringe of a bioprinter. After yielding, the viscosity decreases strongly with increasing shear stress, to around 1–10 Pa s^{−1} endured in the nozzle in the bioprinting process (100–500 s^{−1}). Figure 6.2A shows the shear-thinning of poloxamer 407 and several derivatives used in bioprinting,⁷⁴ whereas 2B shows a Herschel–Bulkley fit to methacrylated poloxamer 407 with fitting parameters $\tau_0 = 389$ Pa, $K = 199$ Pa s^{−1} and $n = 0.22$, indicating strong shear-thinning behaviour. Due to its ideal rheology, poloxamer 407 is a popular material serving several different functions in bioprinting. For example, a methacrylated version has been used for printing of a reinforcing ‘scaffold’ gel, including short degradable oligoester links to tune degradation rates.⁷⁴ It has also been used to create perfusable lumen (vasculature) in printed or moulded ‘blocks’ of gels with cells. First, lines and grids were printed in poloxamer 407 which were then surrounded by gels with encapsulated cells. After crosslinking of this gel, the poloxamer 407 was removed by suction and washing at reduced temperatures, taking advantage of the LCST behaviour to liquefy the support gel (Figure 6.2D). Additionally, LCST behaviour is practical for bubble-free loading of cold solutions into syringes prior to printing. Poloxamer 407 has also been investigated as a bioink for encapsulating cells. The uncrosslinked thermal gel erodes over days when incubated in water (or phosphate buffer solution (PBS) or cell culture medium) and actually shows long-term toxicity, as its amphiphilic nature destabilises the cell membrane.⁷⁵ However, when vinyl-functionalised poloxamer 407 is cross-linked the gels are both stable and non-toxic. As high concentrations of

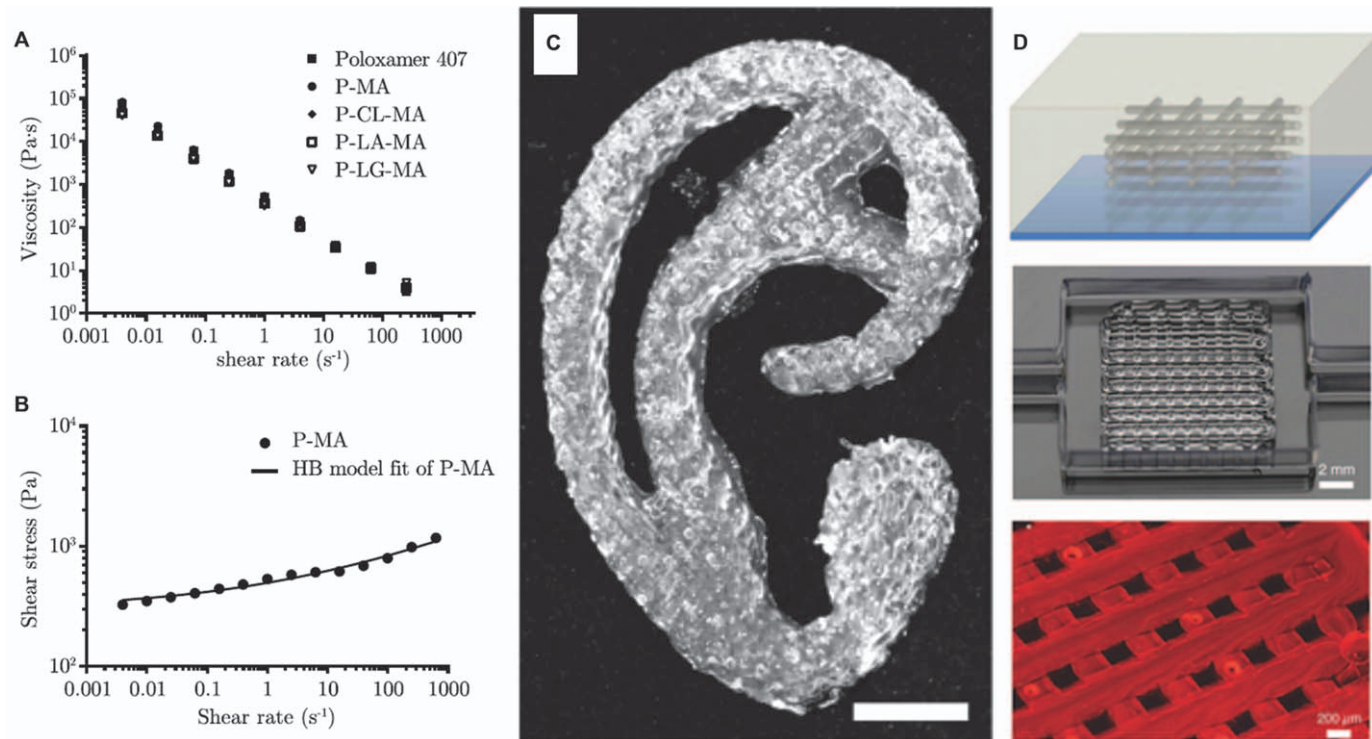


Figure 6.2 Examples of poloxamer 407 used in bioprinting. (A) Shear rate sweep on poloxamer 407 and derivatives (28.6% w/w in PBS) showing shear-thinning behaviour. (B) Fit of Herschel-Bulkley model to shear stress over shear rate of methacrylated poloxamer 407 (P-MA) solution. (C) Model of auricular cartilage printed in P-MA as the reinforcing gel with gelMA (5% w/w) as the bioink (scale bar indicates 10 mm). (D) Schematic illustration, optical image, and fluorescent image of 3D embedded vascular networks that are printed, evacuated, and perfused with a water-soluble fluorescent dye. Parts A, B and C reproduced from ref. 74 with permission from IOP Publishing. Part D reproduced from ref. 96 with permission from John Wiley and Sons, Copyright 2014 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

poloxamer are required for a printable material, the resulting gels are stiff and non-permissive to cells, causing a decrease in viability and function.⁴⁶ However, when the majority of the poloxamer is non-modified and hence not incorporated into the final gel, soft gels with good cytocompatibility can be printed into well-defined shapes (see Section 6.3.3.4 on rheology modifiers).⁴⁷

6.2.3 Solidification

Simply stated, many biofabrication technologies rely on liquid-to-solid transitions to shape biomaterials into well-defined, stable structures. Stereolithography for example, is based on the spatially controlled solidification through photo-initiated crosslinking of a liquid precursor solution, which can include cells.^{54,76} However, the section above demonstrates that bioinks are usually not simple liquids, but more often show complex rheological behaviour to aid in the fabrication process. Although yield stress and shear-thinning effects prevent or delay collapse of the printed structure, additional fortification is usually necessary to obtain a structure that can be manipulated, brought to 37 °C for *in vitro* culture, or eventually, implanted *in vivo*. For hydrogel-based bioinks, usually one or more forms of crosslinking are employed. These can be physical or chemical in nature, each with respective pros and cons. Physical crosslinking is usually achieved under benign conditions, but can be slow (*e.g.*, thermal gelation of gelatine) or resulting in weak gels (*e.g.*, native ECM gels such as collagen type I or Matrigel[®]). Some gels require temperatures which are just outside the physiologically acceptable range to be processed, such as agarose or pure gellan gum (T_{gel} , both around 42 °C). A more suitable physical crosslinking method is by ionic interactions such as calcium-crosslinking of alginate, making this a very popular material for bioprinting. There are other physical crosslinking mechanisms that are still not, or scarcely explored for bioprinting, including stereocomplexation⁷⁷ and DNA hybridisation.⁶⁰

Contrary to most physical crosslinking methods, chemical crosslinking is often fast and effective in obtaining good mechanical properties, but exposure to crosslinker or initiator molecules, or stimuli such as UV irradiation, can have an adverse effect on embedded cells.

In many cases, different forms or degrees of crosslinking are combined in subsequent stages of the bioprinting process, to progress from a more liquid state (for the bioink in the cartridge) to a more solid state (for the final tissue construct). An example of such a combined crosslinking process is the combination of gelMA and gellan gum.^{30,70} The bioink contains sodium chloride at a tailored concentration (0.09 wt%) to achieve mild ionic crosslinking of the gellan gum chains. The resulting weak gel is liquid enough to be extruded through a nozzle but rebuilds directly after extrusion of the ink to prevent sagging of the deposited filament. This results in the bioink exhibiting a yield stress in the range of 1–100 Pa, depending on both gellan and gelMA concentration. Secondly, the gelMA molecules undergo thermally induced physical gelation, reinforcing the structure during printing.

The third step is photo-initiated crosslinking, which gives strength to the structure and prevents it from melting when incubating it at 37 °C.^{30,70}

6.2.4 Final Gel Properties

So far, properties of polymer-based bioinks relevant to the process of fabricating structures have been discussed. However, arguably even more important is the influence these properties have on the embedded cells. Processing parameters, and to some extent material properties, can have an influence on the cells during fabrication. The main concern in this step of the process is to keep the cells alive and to maintain sterility. However, the final properties of the bioink (which has now become the artificial extracellular matrix) have a decisive effect on the fate of the embedded cells upon maturation or application of the tissue model. Affected behaviours include cell survival, metabolic activity, motility, proliferation, cell phenotype, (de)differentiation, and matrix production – both qualitative and quantitative. The desired cell behaviour, and allowable deviations from native tissues, depends largely on the application of the tissue model, as explained in Section 6.3.2 Cell-Supporting Materials.

6.3 Functions of Polymer-based Biomaterials in Biofabrication and 3D Tissue Modelling

In this section we will focus on the function different polymers serve in the biofabrication process, uncoupled from their source or chemical structure. The main properties of the polymers responsible for serving in these specific functions are highlighted.

6.3.1 Scaffolding

6.3.1.1 Pre-fabricated Scaffolds

Similar to scaffolding in the construction industry, in tissue engineering and regenerative medicine a scaffold is a support structure that provides temporary mechanical support. The scaffold is intended to gradually disappear as new tissue is being produced by the cells. The scaffold provides surface area within the pore space for cells to adhere and build tissues, and can further provide biochemical cues. Fabricated scaffolds would be post-processed, sterilised and seeded by filling the pore space with a cell suspension; either manually or using bioreactors to enhance cell seeding efficiency and uniformity.⁷⁸

6.3.1.2 Co-printing of Scaffolds for Biofabrication

To better recapitulate the complex organisation of tissues, 3D bioprinting includes cells in the fabrication process, allowing the placement of different

cell types and materials in different areas in an engineered tissue construct. Although most bioprinting is performed using only the cell-laden hydrogel, it can include a scaffolding component as well. Particularly when the engineered tissues are intended for transplantation, a minimum stiffness and strength are required to withstand the endured physiological loads, which strongly depend on the host tissue. As the scaffolding material will be deposited alongside cell-laden hydrogels, strict requirements exist on the processing conditions including temperature, pH, osmolarity, and presence of cytotoxic components or harmful energy sources such as UV. These limitations render many of the techniques and materials previously employed for the fabrication of scaffolds useless. Nevertheless, the successful co-printing or hybrid printing of scaffolding materials with cell-laden gels has been reported. Wouter Schuurman *et al.* printed ϵ -poly(caprolactone) (PCL) alongside cell-laden alginate, thereby enhancing the constructs' stiffness from several kPa (alginate alone) to several MPa (PCL scaffold with or without alginate). As the mechanical properties of the hybrid constructs is determined mostly by the PCL scaffold, they can easily be tailored by changing filament spacing, orientation and/or thickness.⁷⁹ At the same time, the viability of the cells encapsulated was not significantly affected. After this first report, several other instances of PCL co-printed with cell-laden bioinks have been published, which reinforces its feasibility.^{44,79} PCL has a favourably low melting point and is highly suited as a tissue engineering scaffold material,⁸⁰ however the development of polymers with a larger range of mechanical properties and degradation times (which is 2–3 years for PCL) would be desirable for the purpose of co-printing scaffolding materials alongside bioinks. One demonstration of co-printing using a different scaffolding material employed a hydrogel.⁷⁴ Poloxamer 407, which as stated before is a popular printing material for its excellent rheological properties, was modified with methacrylate end groups allowing for photo-initiated crosslinking after printing, resulting in a rubber-like material suitable for the fabrication of soft tissues. In addition, short polyester links of different monomers were included to tailor degradability from weeks to months (Figure 6.3ABC).⁷⁴

An example of a non-polymer-based material that can be co-printed with bioinks under ambient conditions is self-setting calcium phosphate cement, which has been co-printed with alginate.⁸¹

6.3.2 Cell-supporting

In biofabrication, the cells to be positioned will be suspended in a hydrogel that serves to deliver the cells and provide an initial extracellular matrix. This cell-supporting material is termed bioink (analogous to the ink in book printing), and although it is sometimes also referred to as a scaffold, it serves a different purpose than the scaffolding materials described above. Nevertheless, one of the roles of the cell-supporting bioink is mechanical support. Anchorage-dependent cells need not only ligands to attach to (biochemical

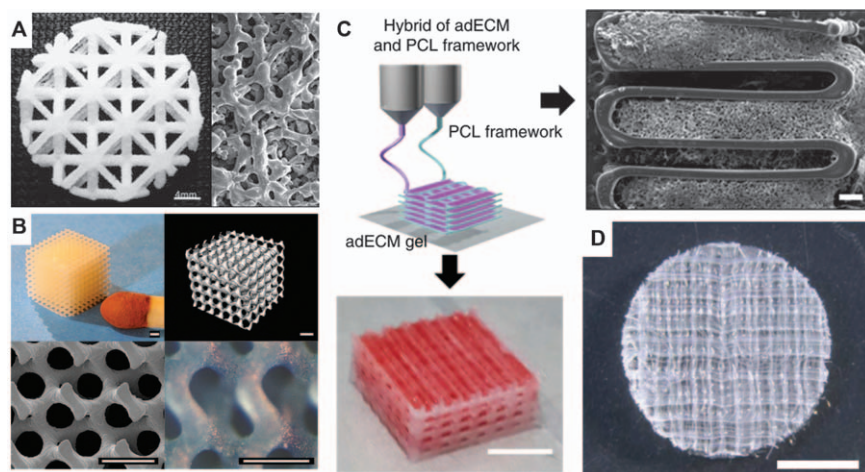


Figure 6.3 Examples of 3D printed scaffolding polymers. (A) PCL scaffold prepared by selective laser sintering (scale bar = 4 mm) with microporous surface visualised by SEM at 100 $\times$ magnification. (B) Gyroid porous scaffold prepared from thermoset poly(D,L-lactide)-dimethacrylate prepared by DLP stereolithography. Scale bar = 0.5 mm. (C) Hybrid printing of adipose-derived extracellular matrix reinforced with a poly(ϵ -caprolactone) framework. (D) Composite of gelMA hydrogel reinforced with a PCL microfiber scaffold prepared by melt electrospinning writing. Part A reproduced from ref. 104 with permission from Elsevier, Copyright 2009 Acta Materialia Inc. Part B reproduced from ref. 105 with permission from Elsevier, Copyright 2009. Part C reproduced from ref. 44 with permission from Nature Publishing Group. Part D reproduced from ref. 106 with permission from Nature Publishing Group.

environment), but also a matrix of appropriate stiffness (mechanical environment). The influence of substrate stiffness on cell behaviour has been extensively studied in 2D,^{82,83} but it also plays a crucial role in 3D.^{84,85} Many other properties of cell-supporting materials are important for their performance, which depend strongly on the application of the fabricated tissue construct.⁸⁶ These applications usually fall within two categories: as *in vitro* models, or for *in vivo* regeneration and repair.

6.3.2.1 In Vitro Models

In vitro tissue models can be used for drug discovery,⁸⁷ personalised drug testing,⁸⁸ studying disease²³ or other biological processes, *e.g.*, fertilisation.⁸⁹ Whilst properties that affect the intended use are of high importance, others are less so. For example, the immunogenicity of used materials is not a problem in most cases, as the *in vitro* model lacks an immune system. Furthermore, degradability of the material is usually not required. Material properties that are important are diffusivity⁹⁰ (of nutrients, drugs and metabolites), stiffness⁹¹ (important for cell survival and differentiation), and

the presence and concentration of the proper biochemical cues⁸² (to maintain the right phenotype). On top of these material requirements, the spatial organisation imposed by the biofabrication technique should improve the performance of the model as compared to a homogenous cell-laden gel; otherwise, the bioprinter would function merely as a pipetting robot.

6.3.2.2 In Vivo Regeneration and Repair

In vivo application of biofabricated tissues, potentially first matured *in vitro*, imposes additional requirements compared to the application as models such as described above. Firstly, upon implantation, neither the material nor other components of the tissue model should elicit an unacceptable immune response. Most polymers have low inherent immunogenicity; however they may contain endotoxins (remnants of bacteria) that can invoke strong immunogenic reactions. Removal of these endotoxins is technically challenging as well as costly. Therefore, the best approach seems to be starting off from low-endotoxin materials (preferably of synthetic origin) and performing all manufacturing steps under aseptic conditions. Secondly, the tissue model should have sufficient mechanical properties (mainly stiffness, strength, toughness and resistance to fatigue) to withstand the continuous cyclic loads endured in the body. Attempts to reinforce hydrogels can hinder cell functioning, in particular, proliferation and matrix formation. Therefore, the functions of mechanical support and cell support can be separated into two distinct materials, a scaffold material and a bioink, which can be co-fabricated with some biofabrication technologies.^{44,79} Eventually, degradation is important as in most cases—the material is intended to serve a temporary function and should gradually disappear as the tissue matures and strengthens.⁹² Polymers in tissue models can degrade either spontaneously (*e.g.*, by hydrolysis of ester, anhydride or amide bonds), or be actively degraded by cell-secreted enzymes or superoxides. The latter occurs for materials with inherent biodegradability such as proteins, or for synthetic materials which have been engineered to include enzyme-cleavable linkages. These approaches have the advantage that cell-free regions within the hydrogels retain their stability and strength, whilst material is degraded selectively around cells to make space for cell proliferation, migration and tissue formation. An example of this is in the culture of tumour spheroids within gelMA gels. It was shown that by inhibiting the expression of matrix metalloproteases (MMPs), which are enzymes that can degrade the proteinaceous matrix surrounding the cell aggregates, the growth of tumour spheroids (both in size and cell number) was slowed down.⁹³

The additional technical requirements for the *in vivo* application described above, along with regulatory barriers, may mean that the routine clinical application of bioprinted tissues is still in the faraway future; whilst *in vitro* models may become commonplace in the coming decade. Yet, promising developments are being made, particularly for tissues of limited complexity, such as skin. One recent study employed bioprinting to produce

skin that was very similar to normal human skin and indistinguishable from engineered skin produced manually and successfully used in the clinic.⁹⁴ Even though in this case the bioprinter was not necessary in creating the organisation required for engineering functional tissue, it did enable production of skin equivalents in an automated and standardised manner, which greatly enhances the clinical potential.

6.3.3 Facilitating Fabrication

Besides forming the 3D tissue model itself, polymer-based biomaterials are also indispensable to help create complex structures, including overhangs, lumen (e.g., vascular networks akin to a blood vessel system) and to fabricate intricate structures using materials that perhaps are biologically favourable but lack the physical properties to be shaped into any form without collapsing.

6.3.3.1 Supporting Structures

The simplest kind of supporting strategy is to print a mould in which a structure with a non-flat base can be printed, such as a hydrogel-based model of a femoral condyle onto a mould of thermoplastic polycaprolactone.⁹⁵ After printing and solidification of the model (in this case by UV curing), the model can be physically removed from the mould. In this case, any material that does not dissolve, nor contain leachable components that can end up in the aqueous hydrogel, will be able to perform the supporting function. One level of complexity up from this example, is when the model has intrinsic overhangs that need to be supported, such as the triple helix structure in Figure 6.4C. Here, the model and support structure are intertwined, and the modelling material (PCL) and supporting material poly(vinyl alcohol) (PVA) are printed alternately in each layer. After the structure is completed, the supporting material is removed through dissolution by a solvent that does not dissolve the modelling material; in this case water. Other possibilities for removal of the support structure are to liquefy it by hydrolysis (e.g., removal of support structures from Stratasys fused deposition modelling (FDM) printers in NaOH), by thermal transitions (e.g., cooling of poloxamer 407 below its LCST⁹⁶), or by chelating ions from a hydrogel (e.g., liquefying alginate gels with citric acid).⁹⁵ Obviously, the removal strategy needs to be compatible with cells if included in the printed model.

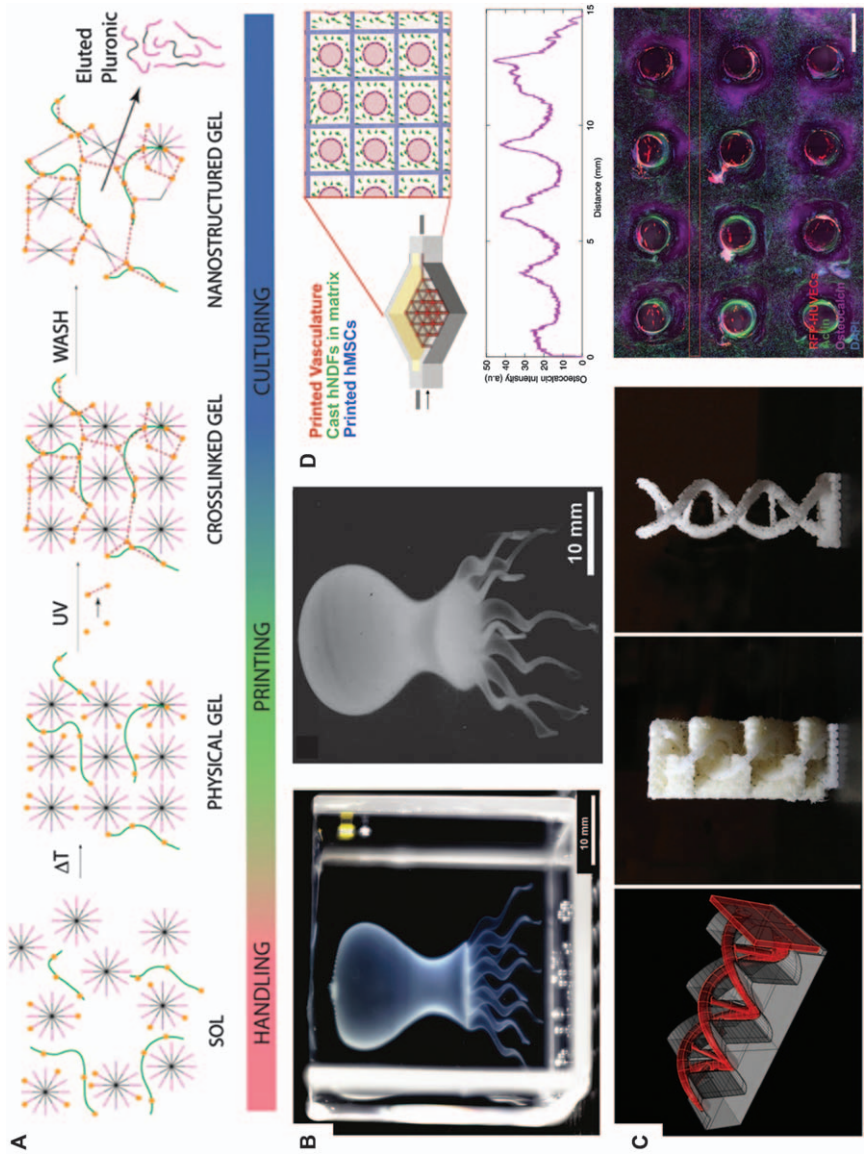
6.3.3.2 Suspension Baths

A recent development in using support materials to print intricate structures from materials that are not inherently printable, is the suspended printing in semi-solid baths (Figure 6.4B). This approach negates the effects of gravity, surface tension and particle diffusion, thereby vastly increasing the

range of materials that can be printed. Additionally, structures will not dehydrate during the printing process, and temperature can be controlled more tightly. The support material, termed a 'granular gel' by the authors, is a colloidal dispersion of highly swollen gel particles of a crosslinked polyacrylic acid copolymer (trade name Carbopol[®] ETD 2020).⁹⁷ As the particles absorb all the surrounding water even at concentrations down to 0.2% (w/v) and fill the whole volume of the bath, the densely packed gel particles behave like a Bingham plastic thus exhibiting yield stress (see Section 6.2.1.3) which has to be overcome to disturb the particle packing. As a result, structures suspended in the granular gel are supported to prevent gravitational collapse as well as surface tension-driven deformation towards spheres. However, the yield stress is sufficiently low to be overcome by a computer-controlled nozzle moving through it, allowing to deposit virtually any material at any position, which will stay in place by the granular gel quickly recovering back from liquid-like to a solid-like state. Using this method, photo-crosslinkable polyvinyl alcohol, polyacrylamide, PEG, HA, alginate, collagen, and even cells without biomaterial were written in this aqueous granular gel.⁹⁷ Additionally, polydimethylsiloxane (PDMS) structures were written in a suspension of PDMS particles diluted with silicone oil. After printing and crosslinking, structures were liberated from the granular gel by dilution and gentle agitation to further disperse the particles and obtain a liquid.⁹⁷ Others concurrently developed an almost identical method which they termed 'free-form reversible embedding of suspended hydrogels (FRESH)'. Here, the suspension medium was a slurry of gelatine particles made in-house with a hand blender, which was used for the direct writing of alginate, collagen, fibrin plus HA, and cells in a mixture of collagen, Matrigel, HA and fibrin. With this type of granular gel, after printing and subsequent crosslinking, the structures were liberated by gentle heating to melt the gelatine particles.¹⁸ Writing in a granular gel medium is not dominated by the rheological behaviour of the ink (written material) but by that of the suspension medium. Therefore, this approach allows for the direct writing of virtually any biomaterial into structures with unsurpassed accuracy and precision, making this a highly promising development for furthering biofabrication and 3D tissue modelling research.

6.3.3.3 Creating Vascular Networks

A persistent challenge in tissue engineering is the supply of oxygen and nutrients to all cells in the engineered tissue, as well as the removal of metabolites. Tissue models cultured *in vitro* often develop a necrotic core, and *in vivo* vascularisation is not fast enough to guarantee timely blood supply to, and survival of all cells within an engineered tissue. Biofabrication technologies create an opportunity to design tissue models including vascular networks that can be perfused with cell culture medium to sustain embedded cells. As the direct printing of hydrogels with small lumen is particularly challenging (the lumen are likely to collapse), strategies have



been developed to print the vascular network using a material that is removed after the gel around it has been stabilised by one of a range of available crosslinking methods.

Kolesky *et al.* used poloxamer 407 for printing their vascular trees. As poloxamer 407 is printed at ambient temperatures (*vs.* 110 °C for ‘sugar glass’) and is possibly less prone to premature dissolution,⁹⁸ it allowed the co-printing of the vascular tree alongside different hydrogels, cell-free or cell-laden, in designed spatial patterns.⁹⁶ After crosslinking of these hydrogels, the poloxamer 407 was liquefied by cooling to below LCST and removed by suction, leaving behind a perfusable vascular network (Figure 6.4D). In a follow-up study, the group produced thick vascularised tissues containing human neonatal dermal fibroblasts throughout the bulk, human mesenchymal stem cells (hMSCs) in specific lines (Figure 6.4D), and human umbilical vein endothelial cells (HUVECs) lining the vascular network that was prepared as before using the fugitive ink.⁹⁹ The vascularised tissues were perfused with growth factors, stimulating the osteogenic differentiation of the hMSCs *in situ*. Increased deposition of osteocalcin (a protein hormone indicating early bone formation) was observed near the lumen through which the growth factor was perfused.

Despite these promising developments, creating vascular networks in 3D tissue models remains a major challenge. This is demonstrated by the fact that the vast majority of cells in the human body reside within a distance of 0.2 mm to the nearest blood supply, which is around the maximum resolution of many fabrication techniques.

6.3.3.4 Rheology Modifier

In many cases, bioinks are formulated using a combination of different polymers to obtain suitable rheological properties for biofabrication. For gelMA for example, the addition of HA increases its viscosity,³³ whilst adding

Figure 6.4 Examples of polymers used as a processing aid to facilitate biofabrication. (A) Poloxamer 407 used as a rheology modifier to enable bioprinting. (B) Model of an octopus written with a photo-crosslinkable PVA-based hydrogel within a granular gel medium (left), and after PVA photo curing and removal of granular gel medium (right). (C) DNA model printed from PCL using water-soluble PVA as a temporary support. (D) Enhanced osteogenic differentiation of within thick vascularised bio-printed tissue, where poloxamer 407 has been used to create open channels in a combined printed/casted hydrogel containing mesenchymal cells and fibroblasts, respectively.

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gellan gum induces a yield stress.³⁰ One will have to consider the impact the addition will have on the final gel properties and behaviour of encapsulated cells, as these added components linger in the gel for prolonged times.

An elegant approach to improve the rheology of an otherwise unprocessable ink is the addition of a rheology modifier that can be easily removed after fabrication. First, a functionalised polymer is mixed with a rheology modifier and printed into a designed structure. Then the structure is stabilised by crosslinking the first polymer (*e.g.*, by photo-initiation) after which the rheology modifier is eluted to yield a more dilute, soft gel that forms a permissive environment for cells to migrate and proliferate (Figure 6.4A). Also for this purpose, the ‘usual suspect’ poloxamer 407 has proven a useful polymer as it shows suitable rheological properties whilst being of sufficiently low molecular weight to be easily eluted (12 kDa, compared to up to 1 MDa for gellan gum for example). A mixture of 3% acrylated and 17% non-modified poloxamer 407 (20% in total) showing favourable rheology for printing was photo-cured, after which the non-modified polymer was washed out of the gel. The resulting soft gel sustained encapsulated chondrocytes in culture at 86% viability, compared to 62% for a stiff gel prepared from just 20% acrylated poloxamer 407.⁴⁷ A similar approach was followed using polymer poly(*N*-isopropylacrylamide) grafted hyaluronan (HA-PNIPAAm) as the rheology modifier with HAMA as the final gel component.³⁷ PNIPAAm is a well-known thermoresponsive polymer that exhibits LCST behaviour like poloxamer 407. Grafting it on HA results in the formation of a printable physical network above LCST due to the collapse of PNIPAAm side groups forming physical crosslinks between the HA chains. However this network breaks down upon cooling for easy removal, only after the HAMA has been photo-cured to induce shape stability.

6.3.4 Sensing

As tissue engineering aims to restore form and function *in vivo*, a desire to restore sensory function may require the integration of sensors and electronics into 3D tissue models. Although this sub-field is very much in its infancy, a notable development was reported by Manno *et al.*, who bio-printed a bionic ear of living cartilage-like tissue with a functional integrated hearing device.¹⁰⁰ Three inks were used: a chondrocyte-laden alginate hydrogel for the cartilage tissue, an electrically conductive silver nanoparticle infused silicone for the inductive coil antenna, and a neat silicone as a support material. After 10 weeks of culture, the printed cell-laden gel had matured into a GAG-rich cartilage tissue with good mechanical properties: stiffness of 112 kPa (a 10× increase during culture period) and physiologically relevant tissue hardness (39–47 kPa). At the same time, the inductive coil antenna proved functional in receiving and transmitting radio signals, and ‘listening’ to stereophonic audio music. This work is the first, and thus far only demonstration of the seamless integration of functional electronics with functional engineered tissue.¹⁰⁰

6.3.5 Actuating

In the tissue engineering for regenerative medicine paradigm, the 3D scaffold (possibly manufactured by 3D printing) is expected to degrade in time to free up space for the growing and maturing tissue. Degradation can proceed either through hydrolysis or through active resorption by the inhabitant cell population. A more active role of the polymer scaffold during the tissue growth and maturation might be beneficial, for example to activate cells to form tissue through mechanical stimulation, or to direct the growth of the tissue. In this light, an interesting recent development is 4D (bio)printing, where a structure is 3D printed in a way that it will change in shape over time (the 4th dimension) after being fabricated. This does not include the action of cells that degrade the biomaterials and make new tissue; by using such a broad definition, any bioprinting or even tissue engineering would be 4D. Neither does it include the response of biomaterials to stimuli such as heat, light or ions in helping to shape tissue models as part of the fabrication process. In this context, by actuating we refer to the capability of biomaterials to perform an action (such as shape change) in a predictable and designed manner after the fabrication process, which has been built into the material. Thus, 'the material is the machine'.

6.3.5.1 Shape Memory Polymers

Shape memory polymers (SMPs) have the capability to store a temporary shape for a long time, and recover to a permanent shape upon receiving a stimulus, most commonly heat. In principle, any crosslinked polymer is an SMP, as the crosslinks determine the permanent shape, and a temporary shape can be stored by bringing the polymer below its glass transition temperature (T_g), effectively vitrifying the temporary shape. Obviously, only polymers with a T_g below or around body temperature will be capable of shape recovery under conditions relevant to tissue models. Besides vitrification, crystallisation can also be used for locking in the temporary shape. The potential of SMPs for biomedical applications is large; however, the application in tissue engineering research is still limited. The use of SMPs has not yet been reported in biofabrication in the strictest definition (*i.e.* including living cells). One report on '4D printing smart biomedical scaffolds' is based on stereolithography fabrication of soybean oil epoxidised acrylate. The crosslinked elastomer recovered at 37 °C from the temporary shape stored by vitrification at −18 °C (Figure 6.5A). The fabricated scaffolds supported the attachment and growth of human bone marrow-derived MSCs.¹⁰¹ Nevertheless, many scaffolds reported before would have shown such shape recovery if the researchers had thought to investigate them. So far, in only one paper the influence of shape recovery on seeded cells was investigated, and it was concluded that the mechanical stimulus imparted by shape recovery was able to influence the shape of cells and nuclei.¹⁰²

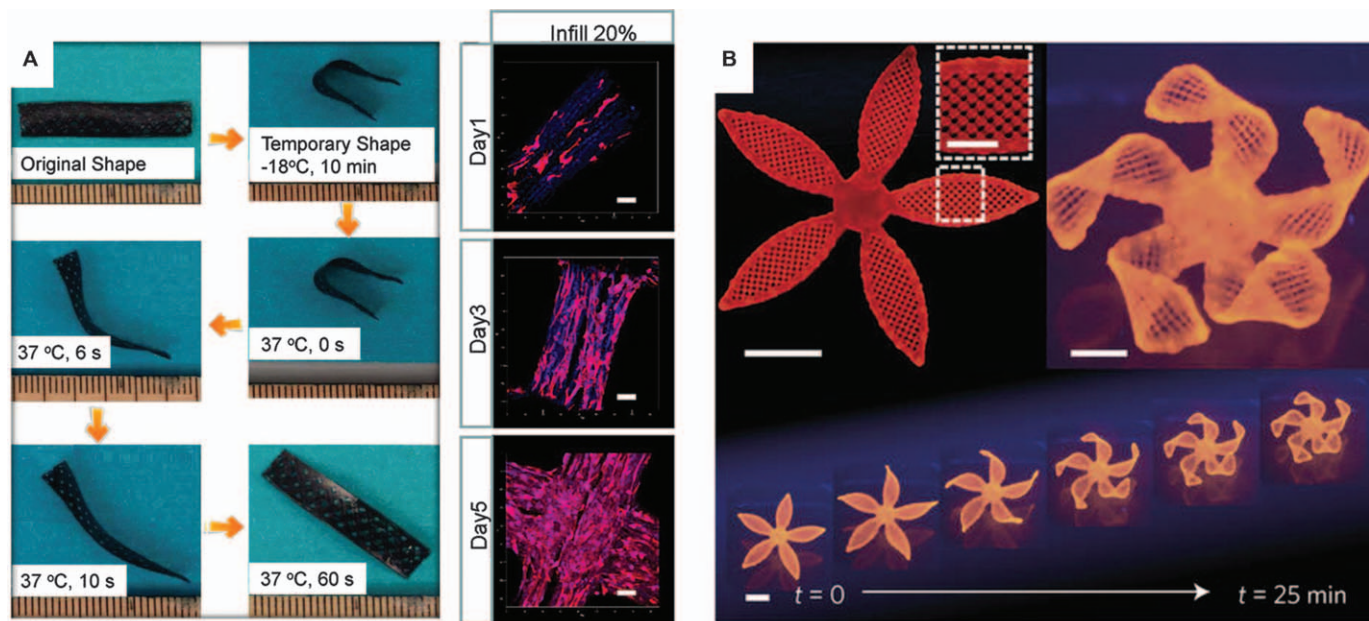


Figure 6.5 Examples of actuating polymers in biofabrication. (A) SMPs based on soybean oil epoxidised acrylate fabricated with stereolithography. Demonstration of shape memory effect (left) and suitability as scaffold for cell culture (right). (B) Designed shape changing of flat-printed hydrogel structures driven by anisotropic swelling of aligned cellulose fibrils embedded within the acrylamide hydrogel, mimicking the opening and closing of flowers. Part A reproduced from ref. 101, <https://dx.doi.org/10.1038/2Fsrep27226>, under the terms of the CC BY 4.0 License <http://creativecommons.org/licenses/by/4.0/>. Part B reprinted from ref. 103 with permission from Nature Publishing Group.

6.3.5.2 Anisotropic Swelling

An intriguing approach to 4D printing is by the extrusion printing of hydrogel structures with embedded cellulose fibrils that cause anisotropic swelling upon immersion in water.¹⁰³ The fibrils (having a large aspect ratio) are aligned in the direction of the printed filament as they are sheared in the printing nozzle. As the fibres resist swelling in the longitudinal direction, the printed filaments swell more in the transverse direction when imbibed in water. By combining fused layers of filaments printed in different directions, the researchers were able to predict the direction and extent of curving of the multi-layered structures upon swelling. Moreover, they could use their mathematical model such to design a flat print path that would lead to the desired curved shape upon swelling, such as the flower in Figure 6.5B.¹⁰³ Although the final hydrogel is biocompatible, the used acrylamide monomers would not allow encapsulation of live cells. Nevertheless, this is an elegant demonstration of the potential of 3D printed structures to adapt to their environment and may be applied in a biofabrication context in the future.

6.4 Summary and Outlook

In this chapter, the indispensable role of polymers in biofabrication and 3D tissue modelling has been discussed. Until now, naturally-derived polymers have been used predominantly for biofabrication, due to their inherent biofunctionality and/or favourable rheological properties, next to the inability to endow synthetic polymers with the appropriate level of functional complexity. However, it can be expected that in time a gradual shift towards synthetic biinks will be observed as knowledge and technology develop. Building on *circa* 10 years of experience, the bioprinting field is starting to recognise and define the properties required for good printability, and a toolbox has been developed which has broadened the range of materials that can be printed, and the geometries that they can be shaped into. The onus is now on biologists and biomedical scientists to define the environment cells require to perform a desired function; such environments can then be created by combining suitable biomaterials with the right biochemical, mechanical and physical properties in a spatially organised manner to fabricate functional 3D tissue models for *in vitro* study or *in vivo* regeneration.

Abbreviations

AM	Additive manufacturing
CAC	Critical aggregation concentration
dECM	Decellularised extracellular matrix
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EHS	Engelbreth–Holm–Swarm sarcoma cells

FDM	Fused deposition modelling
FRESH	Free-form reversible embedding of suspended hydrogels
GAG	Glycosaminoglycan
gelMA	Gelatine-methacryloyl
HA	Hyaluronic acid
HAMA or MeHA	Methacrylated hyaluronic acid
HA-PNIPAAm	Poly(<i>N</i> -isopropylacrylamide) grafted hyaluronan
hMSC	Human mesenchymal stem cell
HUVECs	Human umbilical vein endothelial cells
LCST	Lower critical solution temperature
MMP	Matrix metalloproteinase
MSCs	Mesenchymal stem cells
NHS	<i>N</i> -hydroxysuccinimide
PBS	Phosphate buffer solution
PCL	ϵ -polycaprolactone
PDMS	Polydimethylsiloxane
PEG	Poly(ethylene glycol)
PHPMA	Poly(<i>N</i> -(2-hydroxypropyl) methacrylamide)
PLGA	Poly(lactic- <i>co</i> -glycolic acid)
PNIPAAm	Poly(<i>N</i> -isopropylacrylamide)
PVA	Poly(vinyl alcohol)
RGD	Arginine(R)-glycine(G)-aspartate(D) amino acid sequence using their one letter code
SDS	Sodium dodecyl sulphate
SMP	Shape memory polymer
UCST	Upper critical solution temperature
VEGF	Vascular endothelial growth factor

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CHAPTER 7

Decellularized Tissue Matrix-based 3D Tissue Modeling

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7.1 Introduction

Owing to the limited availability of donors and immune rejection, tissue engineering research is heading towards personalized therapeutic strategies that demand the emulation of the precise structural architecture of native tissues. An appropriate choice of biomaterials is an imperative factor in the fabrication of tissue-engineered constructs, as the biomaterial should replicate the biological and physicochemical properties of the native extracellular matrix (ECM).¹ The matrix usually influences tissue-specific cell phenotypes by managing cell–cell or cell–tissue interactions.² In addition, the matrix should support neo tissue formation.³ Design and fabrication of tissue-engineered constructs include a range of materials, such as proteins, carbohydrates, synthetic polymers, and composite materials of hydrogels

and inorganic compounds.⁴ Natural polymers that are typically isolated from natural sources are attractive because of their hydrated, biocompatible microenvironment, which is amenable to nutrient diffusion and guiding cellular processes (*e.g.*, migration, proliferation, differentiation, and maturation).⁴ However, they are associated with inferior mechanical stability, higher batch-to-batch variations (*e.g.*, molecular weight and structural composition), and the potential risk of pathogen transfer from the source organism.⁵ Similarly, synthetic materials lack biofunctionality or biologic recognition, which is required for inducing underlying signaling pathways, thereby hampering cellular adhesion and resulting in cell death.⁶ Additionally, hydrogel building blocks or chemical compositions should be chosen appropriately to avoid cytotoxicity and associated inflammatory responses.⁷

The ECM of each tissue type is unique in terms of its inherent compositional and ultrastructural support as a result of dynamic reciprocity between cells and the ECM.⁸ This feature makes it difficult to emulate the intricate complexities of typical native ECM in living tissues. Thus, it would be ideal to provide cells with a microenvironmental niche using decellularized ECM (dECM) to fabricate biomimetic 3D scaffolds and improve cell-biomaterial interactions.

dECM consists of glycosaminoglycans (GAGs), fibrous proteins, and remnant growth factors that mimic the native tissue microenvironment and provides a natural chemical milieu to the encapsulated cells.⁹ In particular, the meaning of decellularization is the lysis and elimination of cellular components in tissues *via* chemical, physical, or enzymatic methods while preserving the tissue-specific ECM.¹⁰ In the past two decades, tissue-specific dECM has been used in a variety of ways in tissue engineering-based therapeutic applications, such as whole-organ decellularized matrices,¹¹ biological sheets,¹² injectable hydrogels,¹³ and cell-derived matrices.¹⁴ Reported studies have highlighted the potential advantage of tissue-specific dECM-based matrices with enhanced cellular functionality¹⁵ and complex tissue formation.¹⁶ A major concern still persists in emulating the complex architectural features and arrangement of human native tissues and organs that are comprised of multiple cell types.¹⁷ Thus, there is a paradigm shift focusing on the demand for advanced scaffold fabrication strategies. 3D cell printing, an advanced rapid prototyping technique, has evolved as a promising solution in the fabrication of complex living tissue-specific constructs through precise and defined deposition of a bioink (combination of biomaterial and cells) at the intended location in a layer-by-layer manner.¹⁸ In this chapter, we delineate the structural and functional role of ECMs and we describe the representative methods of tissue/organ decellularization and the conventional applications of dECM-based matrices for 3D tissue modeling. We also focus on the subsequent use of dECM-based matrices for printing of cell-laden constructs or hybrid constructs by extrusion-based 3D printing technology for functional regeneration of damaged or diseased tissues and organs.

7.2 ECM and Its Functions and Components

Cells and their surrounding ECM are the basic constituents of the human body. In a manner of dynamic reciprocity, they contribute to the establishment, maintenance, and regeneration of functional tissues and organs. The ECM is a collection of cellular secretions that functions as structural supports, growth factor reservoirs, adhesive substrates, and signal transducers, and thus modulates the behaviors of local cells (*e.g.*, cellular adhesion, proliferation, migration, differentiation, and apoptosis).¹⁹ Meanwhile, each tissue contains unique ECM in terms of compositions and structures that endow the ECM with tissue-specific biological functions as well as physical and biomechanical properties. Therefore, because of their capacity to emulate the intricacy of the tissue microenvironment, ECM-based biomaterials have been considered as one of the most promising approaches for both *in vivo* and *in vitro* tissue engineering.

7.2.1 Tissue and Organ Variety

Stem cells develop into multiple cellular lineages and evolve into various tissues. Human tissues can be categorized into four tissue types with distinct functions and properties (connective tissue, muscle tissue, nervous tissue, and epithelial tissue). This tissue classification is followed by the concept of organs at the structural level. An organ (*e.g.*, heart, skin, liver, kidney, and eyes) is composed of one or more types of tissues and can perform single or multiple functions.

The specific biochemical composition and distribution of ECM components vary depending on tissue and organ sources. For instance, ECM derived from porcine small intestinal submucosa (SIS) is composed of more than 90% collagen by dry weight with the majority being collagen (Col) type I and Col types III, IV, V, and VI are present in minor amounts.²⁰ In contrast, urinary bladder matrix (UBM) contains the same collagen types as SIS. However, the majority is Col types III and VII, which are important components of epithelial basement membrane. This difference distinguishes UBM from most other ECM scaffold materials.²¹

Other apparent discrepancies between tissues are their physical and mechanical properties. For example, cornea tissues exhibit the most transparent appearance allowing light transmittance, which can be attributed to the characteristics of resident collagen and its homogeneous alignment.²² In addition, static organs, such as the brain and lungs, are made up of the softest tissues because of their low Young's modulus (50–200 Pa), but bone and teeth, which endure high loading, can be several orders of magnitude stiffer (2–4 GPa) (Figure 7.1).²³ This mechanical difference is because softer tissues contain small proportions of fibrous components, such as collagen and elastin, and predominantly consist of proteoglycans. Notably, the mechanical property of a tissue not only imposes structural integrity but also plays a crucial role in regulating cellular behaviors. Many studies have

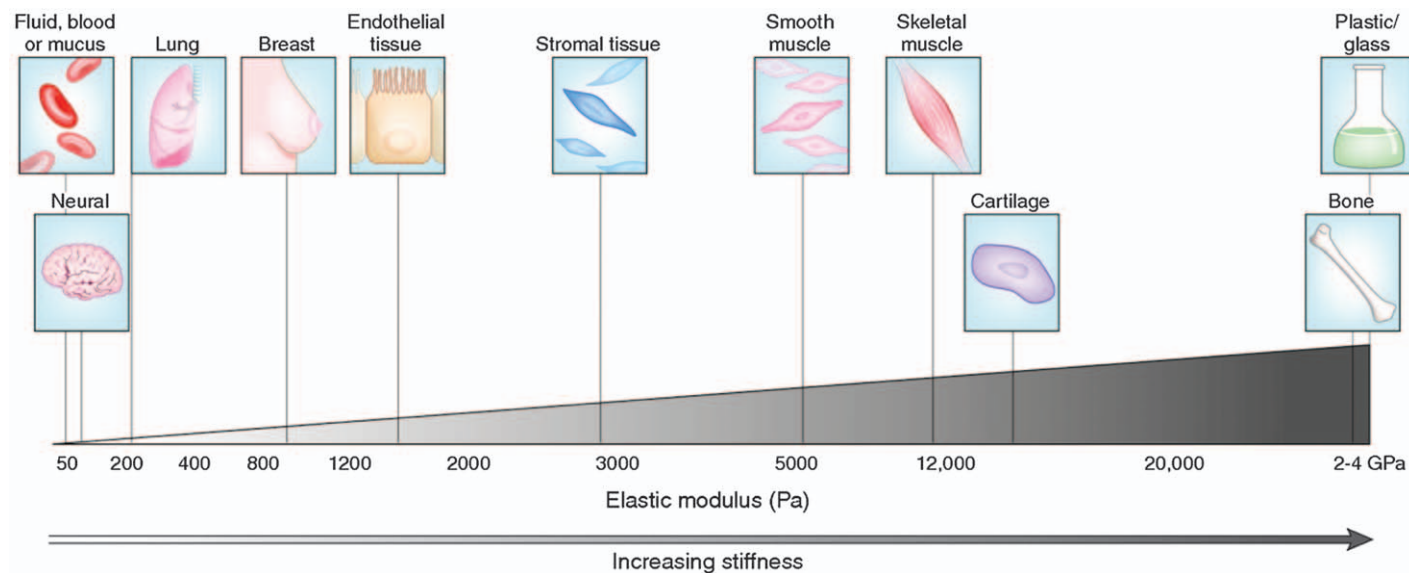


Figure 7.1 Variations in tissue stiffness. The biomechanical properties of a tissue in elastic modulus, measured in pascals (Pa), vary markedly between organs and tissues, and are inherently related to tissue function. Mechanically static tissues such as brain or compliant tissues such as lung exhibit low stiffness, whereas tissues exposed to high mechanical loading, such as bone or skeletal muscle, exhibit elastic moduli with a stiffness that is several orders of magnitude greater.^{23,96} Reproduced from ref. 96 with permission from Springer Nature, Copyright 2009.

demonstrated that cells experience and respond to the mechanical features of their habitats through a process called mechanotransduction, which orchestrates cellular phenotypes and differentiation towards a tissue-specific direction.²⁴ A convincing example is the response of mesenchymal stem cells (MSCs) to substrates with different rigidities: the cells favor a neurogenic path on soft matrices (0.1–1 kPa) but an osteogenic one on a stiff matrix (25–40 kPa).²⁵

7.2.2 Major Elements of the ECM

Despite exhibiting disparate characteristics in different tissues, the ECM is ubiquitous in the human body and mainly consists of several ingredients, including structural proteins (collagen and elastin), proteoglycans, and adhesive glycoproteins (fibronectin and laminin).

Structural proteins, or fibrous proteins, exist in the ECM in the form of fibrils. These proteins, such as collagen and elastin, exhibit robust mechanical strength and enable tissues to withstand tensile, compressive, and repetitive stress without plastic deformation or rupture. Collagen is the most abundant protein found in the ECM and constitutes approximately 30% of the total protein mass in animals.²⁶ It contributes as the main structural ingredient of the ECM, provides tensile strength, manages cell adhesion, supports cell migration, and directs tissue development. The organization and alignment of collagen fibers is regulated by the functional characteristics of the source tissue from which it is derived. Elastin is a highly resilient ECM protein and is found in tissues in the form of fibers, which impart tissues with elasticity to resume their shape under repetitive stretching forces. Abundant elastin is usually present in the medium layer of large elastic blood vessels, such as the aorta, for pressure wave propagation to assist blood flow.²⁷

Proteoglycans comprise a core protein onto which one or more GAG side chains are covalently attached. Owing to the negatively charged GAG chains, proteoglycans are able to sequester water and divalent cations, thereby, performing functions of space-filling, hydration, compression resistance, and lubrication. Aggrecan, a cartilage-specific proteoglycan, is a key ECM component in cartilaginous tissues, such as joints. It confers the ability of dissipating impact force generated during jumping motions through a cycle of water release and absorption.²⁸ On the other hand, the GAG chains can associate and regulate the distribution of biomolecules in the ECM, thereby affecting cellular functions.²⁹

Adhesive glycoproteins are additional ECM components that function as connectors between structural ECM molecules to enhance the network as well as to associate the ECM to cells and soluble bioactive molecules within the matrix. Fibronectin is secreted as a glycoprotein and assembles into fibrillar structures with high molecular weight (approximately 440 kDa). It is composed of two subunits covalently linked with disulfide bonds at their C-termini. Both subunits consist of three modules of repeating units, each with different structures: type I, type II, and type III. These modules

form binding domains associated with various proteins and carbohydrates, such as cell surface receptors (*e.g.*, integrins), collagen and gelatin, heparin, and intermolecular molecules. Therefore, fibronectin plays a critical role in modulating cell behaviors (*e.g.*, adhesion, growth, migration, and differentiation), wound healing, and embryonic development. Laminin comprises α , β , and γ chains that connect at the triple-helical coil-coil region of each chain to form a variety of constructs, such as cross-shaped, Y-shaped, or beam-shaped patterns. Laminin can self-assemble and connect with Col type IV to form an interwoven network in the basement membrane, which is a foundation for most cells and organs. Binding to the cell membrane through integrin receptors, such as dystroglycan and laminin, aids in cellular attachment and differentiation.

7.2.3 Functions of ECM

The most well-known functions of the ECM are the provision of structural support for tissue and organ morphogenesis. The ECM also acts as an interconnected substrate for cellular attachment and migration. However, recent studies have unveiled that ECMs provide biomechanical cues and signals, and also regulate dynamic remodeling.

7.2.3.1 Biomechanical Cues

Tissue-specific ECMs offer unique mechanical-structural properties, including rigidity and topography (spatial organization and orientation), which are critical determinants of cellular behaviors. The importance of rigidity in cell fate decision can be reflected in the case of MSCs in an earlier section. Compared with soft matrices, stiff environments induce integrin clustering, robust focal adhesions, and Rho-MAP kinase activation, leading to improved cellular proliferation and contractility. It has been demonstrated that the spatial organization of ECMs affects the degree of intercellular junction position and magnitude of intra/intercellular forces, reflecting that the ECM arrangements participate in regulating multi-cellular interactions and morphogenesis.³⁰

7.2.3.2 Biochemical Signals

Owing to the presence of multiple protein-adhesive domains, the ECM is capable of localizing and presenting soluble growth to regulate cellular behaviors. For example, many growth factors, such as vascular endothelial growth factors (VEGFs) and fibroblast growth factors, actively adhere to heparan sulfate proteoglycans.³¹ Basement membranes, as another example, maintain apicobasal polarity of epithelial cells and possess tissue-specific composition.²³ The alterations in their biochemical formulation result in changes in physical properties and cellular morphology and behavior, which

can dictate cell proliferation and tumorigenesis by inducing differences in binding activities or spatial distribution of cell surface receptors.

7.2.3.3 Dynamic Remodeling

ECMs are not silent structures. They constantly undergo a remodeling process whereby their components are deposited, degraded, or otherwise modified. The dynamicity of the ECM is an outcome of radical tissue remodeling, such as disease, wound healing and body growth (*e.g.*, development of teeth or skeletons and maturation of the nervous system or reproductive organs). Dynamic remodeling results in changes in ECM organization and composition, by virtue of which cell fate can be regulated. For example, as dermis injury occurs, damaged collagen fibrils and other proteins act as signals for platelets to clot blood at the traumatic site and attract fibroblasts and keratinocytes to induce wound contraction *via* interaction with integrins.³² In return, the fibroblasts deposit matrices that support the migration of other cells towards the wound for regeneration. Moreover, the dynamic degradation and deposition of ECM components serve to create a concentration gradient of proteins and growth factors *via* release and accumulation, a crucial feature determining cell fate. Plenty of research has reported the importance of the concentration gradient of VEGFs in guiding endothelial cells towards angiogenesis.^{33,34}

The native ECM strongly influences the biological responses of cells and plays a crucial role in modulating cellular adhesion, proliferation, migration, and differentiation. Thus, the excellent potential of tissue-specific ECM for *in vivo* regeneration as well as *in vitro* disease modeling makes it a promising biomaterial.

7.3 Approaches for Tissue/Organ Decellularization

The presence of residual cellular and nuclear remnants in dECM may induce *in vitro* cytotoxicity, elicit adverse immune responses *in vivo* upon implantation, and ultimately interfere with the advantages of constructive tissue remodeling in biological scaffolds.⁹ Therefore, the process of decellularization is considered as a critical determinant for successful *in vitro* tissue platform initiation and clinical outcomes. The following sections list different decellularization approaches providing intact ECM niches or substrates for constructive tissue remodeling without inducing adverse immune responses.

7.3.1 Physical Treatments

Physical methods of tissue decellularization involve freezing, direct pressure, osmosis, sonication, and agitation. Rapid freezing of tissues causes formation of intracellular ice crystals, thereby disrupting the cellular membranes and causing cell lysis.³⁵ This protocol has been commonly used for the decellularization of ligamentous³⁶ and nerve tissues.³⁷

However, utmost care should be taken while controlling the rate of temperature change to prevent damaging the ECM ultrastructure by ice formation.³⁸

Direct pressure or mechanical force is used for sparsely and loosely organized ECM,³⁹ which can damage the underlying ultrastructural features and basement membrane integrity of densely arranged ECM.³⁸ Hence, decellularization of urinary bladder, small intestine, skin, and amnion tissues can be performed by coupling mechanical abrasion with hypertonic saline, enzymes, or chelating agents that facilitate efficient detachment of cells from their underlying basement membrane.⁴⁰ Inducing a pressure gradient across tissues during decellularization results in superior preservation of their ultrastructure.⁴¹ Perfusion of hollow tissues like lumen with a transmural pressure gradient (known as convective flow) enables decellularizing agents to flow effectively and forces cell residues out of the ECM. Application of hydrostatic pressure for tissue decellularization requires relatively minimal time and can be more effective than chemically removing the cells from the cornea and blood vessels.^{42,43} However, ultrastructural disruption of the ECM may occur as a result of ice crystal formation, which can be prevented by increasing the temperature.⁴³

Osmotic effect or shock by immersing the tissue or organ of interest alternately in hypotonic and hypertonic solutions through several cycles can also cause cell lysis with minimal changes in matrix molecules and architecture.^{44,45} Mechanical agitation (*e.g.*, magnetic stirring plate, orbital shaker, or low profile roller) and sonication in combination with chemical agents aid in cell lysis and elimination of cellular debris.³⁸

An alternative approach reported for the physical treatment of tissue decellularization is non-thermal irreversible electroporation.⁹ This technique involves the application of microsecond electrical pulses across a tissue, inducing electrical potential across the membrane⁴⁶ and the formation of micropores, causing loss of cell homeostasis and ultimately cell death.⁹ However, this approach is associated with certain limitations, such as the relatively small size of the probes, which restricts the size of the tissue that can be decellularized. In addition, the cell removal mechanism is not clear and has been reported to be immune-mediated, significantly limiting the potential applications of electroporation.⁹

Decellularization by physical treatments requires subsequent post-processing to remove the resulting membranous and intracellular contents from the tissue. Therefore, enzymatic or chemical means of tissue decellularization is necessary for obtaining an acellular tissue that is free of cellular and nuclear debris.

7.3.2 Chemical Treatments

Various types of chemical agents have been reported to decellularize organs and tissues of interest and can be broadly categorized as (i) acids and bases and (ii) ionic or non-ionic detergents.

7.3.2.1 Acids and Bases

Acids and bases are involved in catalyzing the hydrolytic degradation of biomolecules and are highly efficient in solubilizing and removing cellular cytoplasmic and nuclear components.⁹ In addition, they can disinfect the material simultaneously by invading microorganisms and oxidizing microbial enzymes.⁴⁷ Commonly used disinfecting agents, such as peracetic acid, sulfuric acid, and hydrochloric acid can disrupt cell membranes and intracellular organelles to effectively remove the residual nuclear components with minimal damage to the ECM structure and composition.^{48,49} In contrast, bases (*e.g.*, sodium sulfide, calcium hydroxide, and sodium hydroxide) are harsh chemical agents and may remove growth factors entirely from the ECM during decellularization.^{41,50} Bases may cleave the collagen fibrils and disrupt the crosslinking bonds, thereby causing significant reduction in the mechanical properties of the ECM as compared to other chemical and enzymatic agents.⁵¹

7.3.2.2 Detergents

Ionic, non-ionic, and zwitterionic detergents effectively remove cytoplasmic and nuclear material either by individual use or in combination with multiple detergents with increased exposure time.^{52,53}

Non-ionic detergents are widely used because of their relatively mild or less detrimental effects on tissue structure.^{38,54} They enable the separation of lipid–lipid and lipid–protein interactions while preserving the cytoskeletal or cytoplasmic protein–protein interactions and facilitating functional conformation.⁵⁵ One standard example of a widely used non-ionic detergent is Triton X-100 for decellularizing thicker tissues such as valve conduits.⁵⁵ However, decellularization with Triton X-100 has yielded mixed results regarding its effect on cytoplasmic components.

Ionic detergents are also effective in solubilizing both cytoplasmic and nuclear cellular membranes. However, unlike non-ionic detergents, they tend to denature proteins by disrupting protein–protein interactions.⁵⁵ The most commonly reported ionic detergents include sodium dodecyl sulfate (SDS), sodium deoxycholate, and Triton X-200.^{56,57} Unlike Triton X-100, SDS facilitates effective removal of nuclear residues and cytoplasmic proteins from dense tissues and organs, such as the temporomandibular joint and kidney, while maintaining tissue mechanics.^{58,59} Some other studies reported the disruption of collagen with reduction in mechanical properties of certain tissues, whereas no apparent effect was observed on collagen in fairly similar tissues with the same detergent (*e.g.*, tendon *vs.* ligament).^{56,60} Thus, it is very interesting to note that the intensity of effects mediated by SDS and Triton X-100 may vary with the tissue properties. Similarly, non-ionic detergents, such as sodium deoxycholate and Triton X-200, are very effective in eliminating cellular debris. However, they are associated with greater disruption of native tissue architecture compared to SDS. Hence, these

non-ionic detergents are combined with zwitterionic detergents to yield effective decellularization of tissues, such as nerve tissues.^{61,62}

Zwitterionic detergents exhibit properties of both types of detergents and are more likely to denature proteins than non-ionic detergents.³⁹ Examples include the use of 3-[(3-cholamidopropyl) dimethylammonio]-1-propane-sulfonate for vessel decellularization⁴⁸ and sulfobetaine-10 (SB-10) and SB-16 for nerve decellularization.^{61,62}

Taken together, chemical methods for tissue decellularization can effectively remove cellular materials and cell surface antigens. However, they tend to cause irreversible disruption of ECM components that are responsible and highly essential for cellular growth, differentiation, and repair. Additionally, the time of exposure to these chemical agents is a critical factor as it can be disadvantageous for the structural integrity and mechanical properties of dECM grafts. Therefore, tissue decellularization in combination with other (or chemical) treatments is appreciable and should be optimized to elicit minimal or acceptable ECM damage.

7.3.3 Enzymatic Treatments

Enzymatic tissue decellularization involves the use of nucleases, proteases, collagenase, lipase, thermolysin, and α -galactosidase.^{63,64} Trypsin is a serine protease and a highly specific proteolytic enzyme that is commonly used. It functions by cleaving the peptide bonds present on the carbon side of lysine and arginine with maximal enzymatic activity at 37 °C and at a pH of 8.^{9,38} Although removal of cellular and cytoplasmic constituents by trypsin is time-dependent and may require extended incubation periods, trypsin can effectively disrupt the native tissue ultrastructure and enhance the infiltration of subsequent decellularizing agents.⁶⁵ However, collagenous and other ECM proteins, such as elastin show limited resistance to trypsin cleavage or disruption, which in turn may be correlated to the alteration in mechanical properties. Hence, lengthy tissue exposure to trypsin should be restricted.^{57,66}

Additional enzymes such as lipases, a subclass of esterases, aid in catalyzing the hydrolysis of lipidic macromolecules, a process known as delipidation. However, they may not be successful in complete removal of lipids when used alone.⁶⁷ The use of dispase or thermolysin as decellularizing agents *per se* is only effective in dissociating cells from the tissue surface and may require mechanical abrasion for complete removal of cells.⁴⁰ Nucleases such as endonucleases and exonucleases aid in catalyzing the hydrolysis of the internal or mid-sequence bonds and terminal bonds present in ribonucleotide or deoxyribonucleotide chains, respectively, eventually fragmenting RNA or DNA.⁶⁸ Collagenase, in contrast, is used for decellularization based on the intended clinical application where preservation of ultrastructural features or retention of maximum collagen in the resultant ECM may not be a concern.⁹ Other enzymes, such as α -galactosidase can be used to treat decellularized xenogeneic tissues for

reducing the expression of immunogenic cell surface antigen galactose- α -(1,3)-galactose (Gal epitope), although the immunomodulatory effect of the Gal epitope may not hinder in the constructive *in vivo* remodeling of xenogeneic dECM.⁶⁹

Although enzymatic treatments are highly specific in the removal of cells or undesirable ECM residues, they tend to drastically disrupt and alter the native ECM structure, thereby jeopardizing the innate mechanical strength. The maximum enzymatic treatment time can be optimized to alleviate the disruptive effects on the composition and structure of dECM.⁵⁴

7.3.4 Sterilization

Terminal sterilization of the extracted dECM is an equally imperative factor prior to pre-implantation or any *in vitro* analysis. Although the ideal decellularization protocol eliminates nuclear and cellular components, it may not provide sufficient sterilization. Standard clinical sterilization techniques such as dry heating, pressurized steaming, and use of chemicals may cause inevitable protein denaturation.⁷⁰ Several other sterilization techniques such as ethylene oxide exposure,⁷¹ gamma ray or electron beam irradiation,⁷² and solvent treatment⁷³ may damage or alter the ultrastructure and mechanical properties of the ECM.⁷⁴ An alternative method is submersion or perfusion with peracetic acid as it is bactericidal, fungicidal, and sporicidal.⁷⁵ Similarly, supercritical carbon dioxide has been used for multi-log reductions in bacterial and viral loads in porcine dermal ECM accompanied by insignificant changes in its mechanical characteristics relative to those induced by other sterilization methods.⁷⁶ However, this approach is relatively new and further investigation is needed to confirm its capability to sterilize without disrupting the matrix.

One major concern that still persists is the risk of viral contamination while translating both human and animal tissue-derived dECM.⁷⁰ According to reported studies, the only way of addressing this issue so far is by thorough donor screening and proper elimination of residual nucleic acids. Another concern is the microbial contamination of the remaining ECM when subjected to long durations of chemical decellularization.³⁸ Hence, a number of reported protocols have involved the use of antibiotic solutions, such as penicillin, streptomycin, and amphotericin B.^{56,77} Additionally, utmost care should be taken to flush out the residual chemical moieties from the harvested dECM using certain reported assays such as high-performance liquid chromatography-based chemical assay (for measuring detergent concentration),⁷⁸ methylene blue-based non-invasive assay,⁷⁹ and Stains-All assay.⁸⁰ Thus, decellularization and sterilization methods can markedly influence constructive tissue remodeling and its functional outcome as well as the immune response.

7.3.5 Evaluation

Preservation of the innate ultrastructure and biochemical composition of the ECM during tissue decellularization is an eminent factor. A number of

approaches have been reported to quantitatively and qualitatively determine the efficiency of decellularization. Based upon the findings of reported studies, a minimal criterion has been set as a benchmark to satisfy the intent of decellularization: (1) <50 ng of dsDNA per mg dry weight, (2) <200 base-pair (bp) DNA fragment length, and (3) absence or inadequate visibility of nuclear materials and dECM components unveiled by histological and immunohistochemical analysis.^{9,17,38}

Additionally, ECM components can be quantified by spectroscopy-based approaches such as enzyme-linked immunosorbent assay.⁵ Furthermore mechanical analysis can provide general insight into the integrity of structural proteins responsible for the biomechanical properties of native ECM.³⁸

Altogether, the terminal aim of decellularization should focus on effective removal of immunogenic cellular and nuclear residues by preserving the structural integrity and physicochemical properties of the harvested dECM. Moreover, it is indispensable to efficiently sterilize dECM-based biologic scaffolds to obtain a material free of endotoxins and viral or bacterial components.

7.4 Applications in 3D Tissue Modeling

7.4.1 Tissue Modeling Using Conventional Tissue Engineering Methods

In recent decades, dECM in various forms has received increasing attention for the repair and regeneration of various tissues of interest. The conventional approaches of applying dECM are broadly classified into tissue/organ scaffolds, dECM-based biological sheets, and dECM-based injectable hydrogels (Figure 7.2).⁸¹

Choi and colleagues reported a tissue-engineered cornea that provided well-aligned collagen fibrils and showed similar structures comparable to those of native cornea.¹² The expression of specific markers such as ZO-1, connexin 45, and Na^+/K^+ -ATPase was evident 14 days after repopulating the decellularized cornea matrix with corneal endothelial cells. Mechanical analysis demonstrated similar behavior for both native cornea and decellularized stroma with ultimate tensile strengths of 10.6 ± 0.7 and 10.2 ± 2.0 MPa, respectively. The results indicate that decellularization could effectively remove cellular components while preserving both ECM architecture and mechanical properties. Human kidney cortex-derived dECM was reported as a suitable hydrogel for use in in-flow-directed microphysiological systems and supported the function of human kidney peritubular microvascular endothelial cells.⁸² However, inferior mechanical properties still remained a major concern for specific applications.

In order to overcome this limitation, another interesting approach is the use of cell-derived ECM showing the potential to improve cellular responses and targeted differentiation of stem cells. Thibault and colleagues demonstrated the differentiation of rat marrow stromal cells into bone with

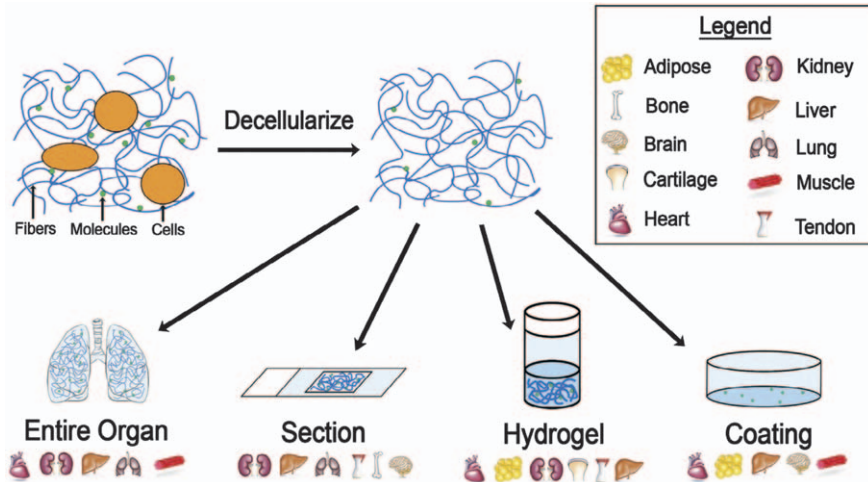


Figure 7.2 Decellularized tissue materials made from various tissues. After removing the cellular contents of the native tissue, the extracellular matrix remains and can be left unprocessed as an entire organ, or be processed into a section, hydrogel, or coating. The tissues that have been processed for the purpose of testing cell differentiation effects are shown below each material category. Reproduced from ref. 81 with permission from Elsevier, Copyright 2016.

significantly enhanced deposition of mineralized matrix when cultured on polycaprolactone (PCL) scaffolds containing cell-laden bone-like ECM.⁸³ However, emulation of the architectural complexity of native tissues remains a major concern. Pati and colleagues reported the improvement in the bioactivity of 3D-printed synthetic scaffolds (composite of PCL, poly(lactic-co-glycolic acid) (PLGA), and β -tricalcium phosphate (β -TCP)) by ornamenting them with mineralized ECM secreted by human nasal inferior turbinate tissue-derived mesenchymal stromal cells (hTMSCs).¹⁴ The hTMSC-derived ECM-laid 3D-printed scaffolds supported osteoblastic differentiation of stem cells *in vitro* with increased expression of osteoblast-specific transcription factors, such as RUNX2, alkaline phosphatase, osteocalcin, and osteopontin.

7.4.2 Tissue Modeling Using 3D Cell Printing of dECM-based Bioink

The standard conventional approaches of dECM-based materials demonstrated appreciable outcomes. However, they suffer from unavoidable structural drawbacks such as lack of tailored microgeometry restricting diffusion of oxygen and nutrients until supported by a vascular network.^{84,85} Thus, in order to better emulate the structural architecture or features of the native tissue, the focus is gradually shifting towards extrusion-based 3D cell printing of dECM.

Extrusion-based 3D cell printing is capable of utilizing a large range of biomaterials and requires bioink to achieve successful fabrication and retention of cellular activities. The first step of applying dECM in 3D cell printing is to formulate applicable bioinks. In general, the desired bioink should possess (1) shear thinning rheological behavior to reduce the hazards on cell viability caused by shear stress, (2) satisfying printability to enable the extrusion-deposition process, (3) sufficient modulus to maintain the pre-designed shape, and (4) cell-friendly gelation mechanism giving rise to sol-gel transition. dECM-based hydrogels display thermoresponsive behavior, exhibiting a liquid phase at low temperature (10 °C) and transforming into a gel at physiological temperature (37 °C).⁸⁶ Pati and colleagues utilized the thermoresponsive phenomenon of dECM-based materials to print tissue analogs. Various types of tissues such as fat, cartilage, and heart tissues were demonstrated to print cell-laden constructs with engineered porosity mimicking the native tissue microenvironment, resulting in multi-lineage targeted differentiation of stem cells (Figure 7.3A).⁸⁶ It involved the co-deposition of an open porous structure of PCL framework as a supportive material together with the specific cell-laden dECM hydrogel according to the intended application, where the printing technology could print feature sizes either at 100 or 200 μm . Moreover, the rheological behavior of each dECM at the same concentration revealed the different and distinctive nature of the bioinks from each other. In addition, the encapsulated human inferior turbinate tissue-derived mesenchymal stromal cells showed high cell viability post-printing, followed by significant upregulation of tissue-specific genes. As a follow-up study, the same group generated 3D-printed dome-shaped adipose tissue constructs using a decellularized adipose tissue (DAT) matrix bioink encapsulated with human adipose tissue-derived MSCs. PCL was used as a supportive framework in order to evaluate its efficacy for soft tissue regeneration *in vivo* (Figure 7.3B).⁸⁷

Higher expressions of adipogenic markers (PPAR γ and Col type IV) were induced without additional supplemented adipogenic factors. Chronic inflammation or cytotoxicity was not observed post-implantation, while positive tissue infiltration and constructive tissue remodeling were observed, leading to more adipose tissue formation than the non-printed DAT gel. A more recently reported study demonstrated the fabrication of a 3D cell-printed pre-vascularized cardiac patch using pig heart-derived dECM (hdECM) in combination with multiple cell types including human cardiac progenitor cells and MSCs (Figure 7.4A).⁸⁸ Upon *in vivo* implantation in a rat myocardial infarction model, enhanced cardiac function and cellular infiltration into the area of infarction was evident with improved vascularization as well as reduced cardiac hypertrophy and fibrosis. Choi and colleagues derived skeletal muscle dECM-based hydrogels facilitating the 3D cell printing of functional skeletal muscle constructs, demonstrating significant enhancement in cellular alignment, subsequent expression of myogenic genes (Myf5, MyoG, MyoD, and MHC), and increased myotube

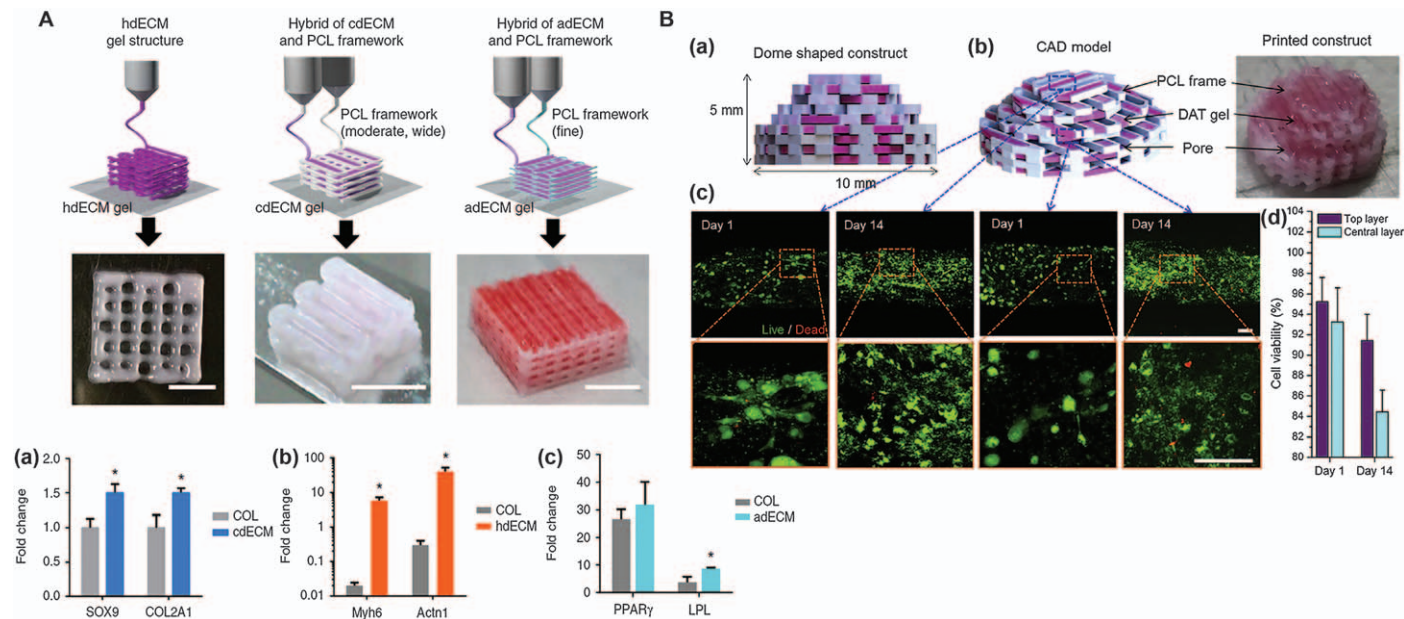


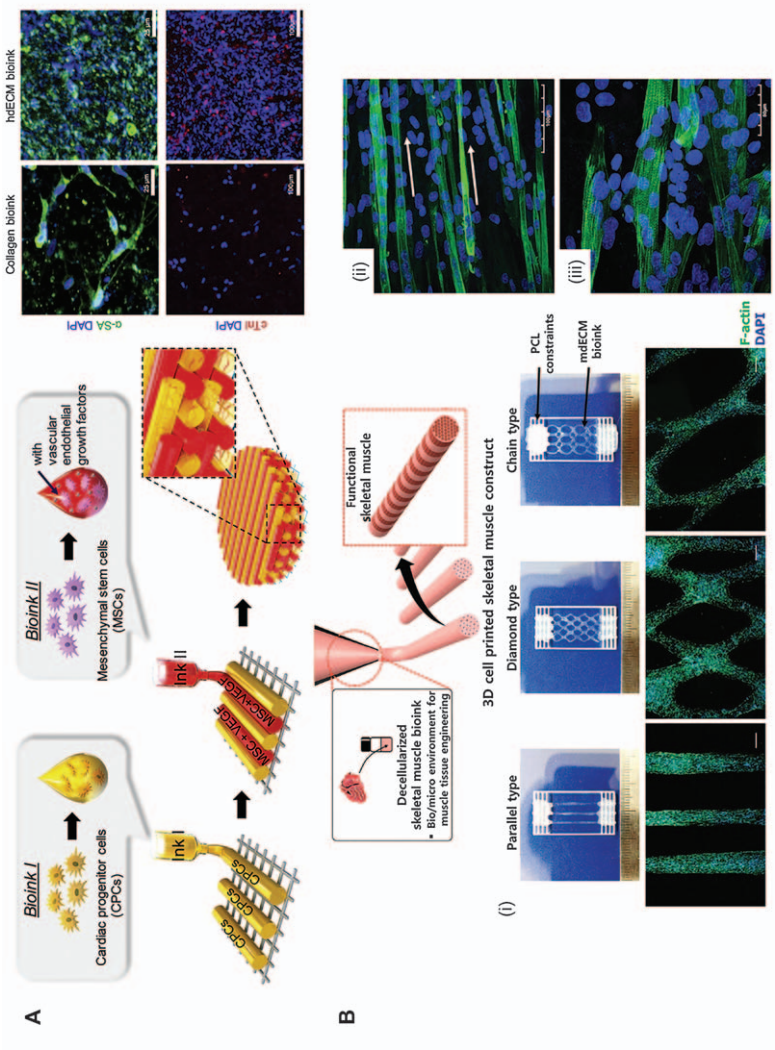
Figure 7.3 Applications in 3D tissue modeling. (A) Heart, cartilage, and adipose tissue construct were printed with heart dECM, cartilage dECM and adipose dECM, respectively (scale bar, 5 mm). Gene expression analysis for (a) chondrogenic (SOX9 and COL2A1), (b) cardiogenic (Myh6 and Actn1) and (c) adipogenic (PPAR γ and LPL) in COL and particular dECM. (B) Fabrication of dome-shaped construct using DAT gel and PCL as framework (a, b). Confocal images showing cell viability (c) and its quantitative evaluation (d) revealing >90% and 80% viability in top and inner layer, respectively. (Scale bar, 100 μ m, Data are expressed as mean $\pm$ s.d.; * p < 0.05.)

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formation compared with a collagen-based construct (Figure 7.4B).⁸⁹ The latest development of a liver dECM bioink reported by Lee and colleagues demonstrated significantly improved functions of encapsulated human hepatocellular carcinoma (HepG2) cell lines and human bone marrow-derived MSCs within the printed liver dECM bioink as compared to the collagen bioink.⁹⁰

The limited mechanical properties and printability of dECM place hurdles for its applications in 3D cell printing. Researchers have struggled to improve these performances in various ways. Gao and colleagues formulated a hybrid bioink composed of 3% dECM extracted from porcine aortic tissue and 2% (w/v) sodium alginate, which provides cell-favorable microenvironments and thus exhibits superior performance to Col type I in governing cellular activities of endothelial progenitor cells.⁹¹ In addition, relying on the ionic gelation of alginate under Ca^{2+} treatment, the hybrid bioink facilitates a direct fabrication of cell/drug-laden vascular structures using a core (shell) nozzle. Furthermore, Jang and colleagues demonstrated that cardiac-derived dECM bioink can be pre-gelled in an easy, versatile, and biocompatible two-step process to improve its printability.⁹² The physical and rheological tailoring of the hDECm bioink was carried out based on thermal and chemical crosslinking using vitamin B2 (VB2) and UVA irradiation during the cell printing process. Specifically, the two-step crosslinking procedure enhanced the stiffness of the VB2-mixed hDECm gel by almost 33 times compared to that of the control hDECm bioink, making it similar to that of native cardiac tissue. Furthermore, it resulted in increased mRNA expression of cardiac-specific genes (GATA4, Nkx 2.5, MEF2C, and cTnI) related to differentiation. Following a different strategy, Skardal and colleagues in the same year developed modular hyaluronic acid- and gelatin-based hydrogels supplemented with dECM harvested from porcine liver and cardiac and skeletal muscle.⁹³ The authors followed a two-step crosslinking procedure to obtain printable bioinks with varied stiffness ranging from 100 Pa to 20 kPa, thereby enabling the prospect of mimicking the mechanical characteristics of different tissues in the body. Similarly, Visser and colleagues created crosslinkable hydrogels by covalent incorporation of dECM materials from cartilage, meniscus, and tendon tissues in versatile and printable GelMA bioinks.⁹⁴ Ahn and colleagues printed skin-derived dECM using a 3D cell printing system equipped with heating modules for precise deposition of cell-laden constructs, thereby facilitating better printability and cell viability.⁹⁵

Overall, 3D cell printing of dECM constructs with interconnected pore networks would provide a 3D hierarchy enabling increased cell-cell and cell-material signaling process for tissue maturation. The accumulative data regarding tissue-derived dECM highlighted that the microenvironment within the bioink plays a determinant role in cellular behavior and functionality. In addition to *in vivo* applications, we can envision the potentials of dECM bioink-based 3D cell printing in establishing *in vitro* tissue models to study biological mechanisms and pathologies, as well as to facilitate the



advances of medical care. Nevertheless, only few studies have been reported and dECM bioinks still retain the disadvantage of insufficient or inferior mechanical stability as the solubilized dECM loses its native structure and mechanical strength during bioink preparation.

7.5 Conclusion and Future Perspectives

The ECM plays an irreplaceable role in mediating cellular activity and functions by providing intricate biochemical and biophysical cues in dynamic manners. Hence, dECM has been considered an optimal biomaterial for tissue engineering because it can inherit compositional and structural features from natural ECM and provide cells with a microenvironment similar to their native habitats. Such advantages have attracted many efforts in the development and optimization of the decellularization process to acquire ideal dECM materials for tissue engineering applications. Although conventional approaches, such as sheets and injectable hydrogels, have achieved considerable outcomes for tissue regeneration, these methods are unable to produce a biomimetic tissue construct with heterogeneous structures and multiple cell types. In contrast, 3D cell printing technologies have great potential in fabricating tissue equivalents because of their ability to precisely control the localization of multiple materials and cells. However, inadequate printability and weak physical stability are major hurdles limiting the full utilization of 3D cell printing techniques and should be regarded as a crucial problem to be solved in the future. Other than merely relying on the merits of dECM, more in-depth analysis should be contributed to unveil the key determinants (*e.g.*, unique biochemical cues, biomechanical signals, and topological features) inspiring cells towards tissue-specific lineages. The outcomes would significantly enrich our knowledge of tissue physiology and morphogenesis, and accelerate the development of *in vitro* tissue models to investigate disease mechanisms and evaluate therapeutic drug efficacy for human medical/health care.

Figure 7.4 Applications in 3D tissue modeling. (A) Illustration of pre-vascularized stem cell patch including multiple cell-laden bioinks and supporting PCL polymer. Immunofluorescence staining against α -sarcomeric actin (green) and cardiac troponin I (red) on day 7. (B) Schematic illustration for fabrication of a 3D cell-printed skeletal muscle construct using a muscle-derived dECM bioink: 3D cell-printed muscle construct with controlled architecture (i), immunofluorescent images of myotubes in muscle constructs at day 14 and their morphometric analysis showing muscle bundles in green and nuclei in blue with magnifications of 40 $\times$ (ii) and 60 $\times$ (iii).

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CHAPTER 8

3D Tissue Modelling of the Central Nervous System

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8.1 Introduction

The central nervous system in vertebrates consists of the brain and spinal cord.¹ The brain controls the complex behaviour of vertebrates and performs a comprehensive set of functions. Specifically, the human brain consists of about 100 billion neurons and ten times more glial cells such as astrocytes, oligodendrocytes, and microglia.² In addition, endothelial cells of the blood vessels and ependymal cells of the ventricles exist in the brain tissue.^{3,4} Therefore, various and numerous cells are represented in the brain. Interestingly, these cells are not randomly arranged, but form delicate and complex structures. Two representative structures of the complex brain are the neural circuits and the blood-brain barrier.^{5,6}

In vitro studies were conventionally performed in a two-dimensional (2D) environment such as a petri dish or a coverslip. These environments were different from the *in vivo* environment: a three-dimensional (3D) space. In order to overcome the limitation of two-dimensional environment, 3D cell

culture methods using ECM hydrogel or cell spheroid were developed that are similar to the *in vivo* environment, but it is difficult to control and observe the tissue.¹³ Organ-on-a-chip using microfluidics arose as a new method for closer reconstruction of organs, achieving a new perspective that cannot be revealed in a conventional cell culture environment. Naturally, there have been many attempts to mimic the brain in the microfluidic platform, overcoming some limitations of previous methods.

In this chapter, we introduce the recapitulation of 3D brain tissue on a microfluidic platform. Reconstruction of specific features of the brain rather than entire features is a better strategy because of the complexity of the brain. Therefore, two important features of the brain in the 3D microfluidic platform were covered: the neural circuit and the blood–brain barrier.^{14,15} These two models reconstructed both the structural and functional features of the *in vivo* brain. For the *in vitro* 3D neural circuit, after changing the internal density of the ECM substrate where neurons were cultured, axons were assembled and fasciculated on a soft substrate, forming an axon bundle structure. Also, synapses between two neuronal groups were formed through the axon bundle. For the *in vitro* 3D blood–brain barrier (BBB), a suitable culture medium for vascular network and astrocytes should be provided. In these conditions, the BBB formed on the platform with a similar level of permeability to the *in vivo* BBB.

8.2 Reconstruction of a 3D Neural Circuit in a Microfluidic Device

8.2.1 Introduction

The neural circuit consists of neurons. Each neuron forms synapses with hundreds of other neurons, forming a neural network that exchanges complex information.⁷ Neuronal connections in the neural network are not random. Neuron groups that constitute each region of the brain accurately project axons into the destination neuron group. There are two kinds of methods in which the orientation of the neural networks can be established during the development of the brain. One is chemical signalling, typically netrin attracting axons and slit repulsive axons.^{8,9} In addition to this chemo-gradient method, there is another method where the orientation of the axons is determined by the stiffness of the tissue substrate. This method is based on the mechanosensing of axons that grow from a stiff substrate to a soft substrate.¹⁰ The axons of the neural network have specific structural features: axons are fasciculated rather than individually located until they reach their destination. When the axon bundle arrives at its destination, it is defasciculated and forms a synapse at the appropriate neurons.¹¹ Based on these structural features, neural signals can be successfully transmitted through synapses in the neural network.

8.2.2 Methods for *In Vitro* 3D Neural Circuit Platform

8.2.2.1 Microfluidic Platform Fabrication

The microfluidic platform was fabricated by soft lithography, which utilized a master device to create multiple soft molds. The master mold wafer was fabricated by photolithography with an SU-8 negative photoresist (MicroChem, USA) and a silicon wafer (Unisill Wafer, Korea). A 10:1 (w/w) Polydimethylsiloxane (PDMS, Sylgard 184, Dow Corning) and curing agent mixture was poured onto the master and degassed in a vacuum chamber for the removal of bubbles, and then thermally cured to obtain negative replica molds. Sterilized glass coverslips and PDMS microfluidic platforms were treated with air plasma to bond permanently to each other. After bonding, the microfluidic platforms were put in an 80 °C dry oven for at least 48 hours to make the channel surfaces hydrophobic. The devices were sterilized by UV irradiation before the experiment.

8.2.2.2 Deformation of Matrigel in the Microfluidic Platform

Matrigel in the liquid state was injected into the first ECM hydrogel channel and solidified at room temperature after 10 min. This step was repeated for the second and third ECM hydrogel channels. To make both medium channel surfaces hydrophilic, the medium was filled into the reservoirs and pulled from the opposite reservoir by suction. More medium filled the plastic reservoirs until the water level was at 12 mm and removed the medium completely from the opposite medium channel without the plastic reservoirs. The microfluidic devices were kept in a 37 °C incubator for 3 hours until Matrigel was pushed out to the medium channel.

8.2.2.3 Primary Neural Cell Preparation and Plating in the Microfluidic Platform

Rat cortical neurons were prepared from a Sprague–Dawley embryonic rat (E17). The rat embryo cortexes were incubated for 10 min at 37 °C in a trypsin-EDTA solution (Gibco, USA). After incubation, the supernatants were removed and Dulbecco's modified Eagle's medium (Gibco, USA) containing 10% Fetal Bovine Serum was added to stop the trypsin reaction. The supernatants were removed again before neurobasal medium (Invitrogen) containing 2% B27 supplement (Invitrogen, USA), 0.25% GlutaMax (Invitrogen), and 1% penicillin–streptomycin (Invitrogen, USA) were added. The cortexes were triturated with a glass pipet. Finally, cell suspension was filtered through a cell strainer and the required concentration was made by adding neurobasal medium. Cell suspension was placed in a 50 µL microfluidic platform. The microfluidic platforms were kept in a 37 °C incubator that tilts 90° to settle the cells down to the hydrogel surface. The microfluidic platforms were incubated at 37 °C and 5% CO₂. The medium was changed to fresh neurobasal medium every 48 hours.

8.2.2.4 Immunostaining

Cells were fixed in 4% paraformaldehyde and permeated with 1.0% Triton X-100 in PBS for 10 min. After these steps, cells were treated with a blocking solution of 10% (v/v) fetal bovine serum, 0.5% (v/v) Triton X-100, and 0.2% (w/v) gelatin in 0.1 M PBS at room temperature for 2 hours. Primary antibodies were diluted in antibody solution: 5% (v/v) fetal bovine serum, 0.5% (v/v) Triton X-100, and 0.2% (wt/v) gelatin in 0.1 M PBS and incubated at 4 °C for 2 days. Species-specific secondary antibodies were incubated for 1 day at 4 °C. The nuclei were visualized with 2 $\mu\text{g mL}^{-1}$ Hoechst (Molecular Probes, USA) staining for 2 hours.

8.2.3 Results and Discussion of *In Vitro* 3D Neural Circuit Platform

8.2.3.1 Deformation of Matrigel and Formation of Axon Bundle

The microfluidic platform for the formation of an axon bundle or neural circuit consisted of three hydrogel channels and media channels on both sides with micro-posts (see Figure 8.1a). Liquid Matrigel was patterned on hydrogel channels with micro-posts. After the Matrigel was stored in a 37 °C incubator for 30 minutes with no external force, Matrigel was fully gelled and the density inside the gelled Matrigel was isometric in all directions.

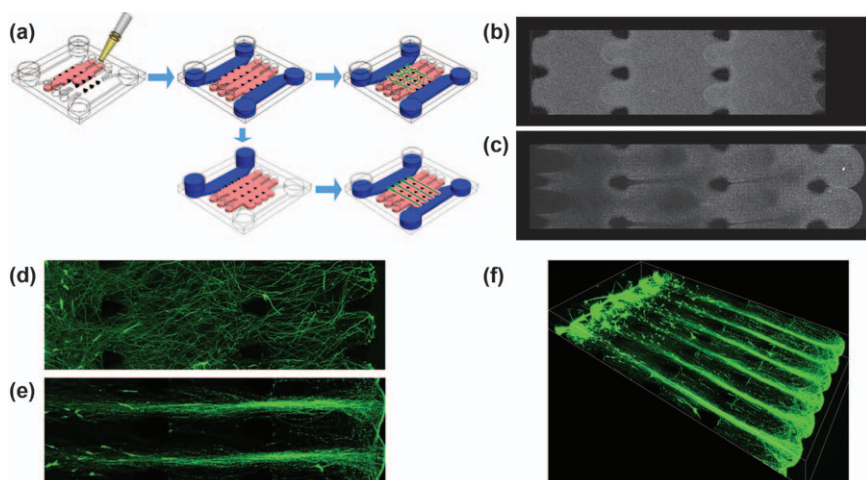


Figure 8.1 (a) Schematic of microfluidic platform for axon bundle formation. (b) Randomly cross-linked Matrigel. Collagen type IV density distribution is isometric. (c) Deformed Matrigel, collagen type IV density distribution is anisometric. (d) Neurites at the randomly cross-linked Matrigel are spread in all directions. (e) Neurites at the deformed Matrigel gather and form bundle shape. (f) The bundle is a three-dimensional shape that is maintained not only in the xy plane but also in the z-axis direction. Reproduced with permission from ref. 14, Copyright 2015 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

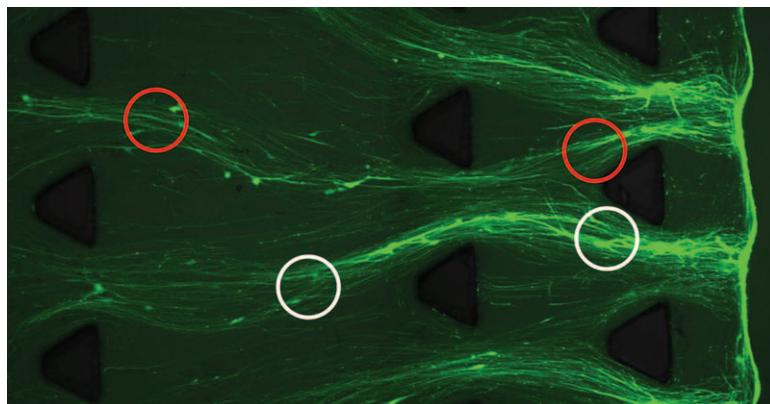


Figure 8.2 Axon bundles in staggered arrays of micro-posts. The axon bundles on either side of each other merge into one place in the next micro-post array, then separate in the following micro-post array, keeping the original shape.

On the other hand, after Matrigel was stored at room temperature for 10 minutes, only the surface of Matrigel was gelled and inner Matrigel was still liquid. That is, the surface of Matrigel did not flow out during introduction of the medium while the inner area of Matrigel could be deformed. The formation of an axon bundle was performed by application of external force on different states of Matrigel. A different hydrostatic pressure to both sides of the Matrigel was applied, increasing the medium level in only one medium channel, then this is stored in the incubator for 3 hours. During this time, Matrigel became fully gelled and deformed by hydrostatic pressure. After 3 hours, Matrigel was partially pushed out to the media channel from the hydrogel patterning channel. The density inside the deformed Matrigel had an anisotropic pattern, which was repeatedly divided into high density regions and low-density regions (see Figure 8.1b and c). Cortical neurons were attached to the surface of unmodified Matrigel and modified Matrigel, respectively. The axons of neurons cultured in unmodified Matrigel grew disorderly inside Matrigel, while the axons in the deformed Matrigel grew into bundles in the low-density region of Matrigel. The convergence of the axons according to Matrigel deformation was seen not only in the x - y plane but also in the z -axis direction (see Figure 8.1d, e and f).

If the micro-posts of the hydrogel patterning channels are not parallel but staggered, the axon bundles were temporarily together between the micro-posts, but separate again in the following micro-post array (see Figure 8.2).

8.2.3.2 Formation of the Neural Circuit through the Addition of Post-synaptic Neuron Group

The axon bundle cultured in deformed Matrigel cannot be regarded as a complete neural circuit due to the absence of a post-synaptic neuron.

A post-synaptic neuron group is cultured at the end of the axon bundle for a completed neural circuit. Therefore, the design of the microfluidic platform was modified to locate the post-synaptic neuron group by arranging one micro-post array next to the micro-post array forming the axon bundle (see Figure 8.3a). Post-synaptic neuron groups were cultured in collagen type 1 because the antibodies were not completely diffused inside Matrigel. After the axon bundle entered the post-synaptic neuron group, it dispersed into individual axons (see Figure 8.3b and c). Synaptophysin—as a pre-synaptic marker—and PSD-95—as a post-synaptic marker—reveal a complete synapse, distinguishing pre-synapse and post-synapse. There is a high probability that neurons in the post-synaptic neuron group will form synapses with each other at the central or right area of the post-synaptic neuron plated micro-channel. Therefore, observation of the synapse was performed at the point where the axon bundle first met the post-synaptic neuron group. Synapses in which pre-synaptic markers and post-synaptic markers were expressed together were found in the regions (see Figure 8.3d).

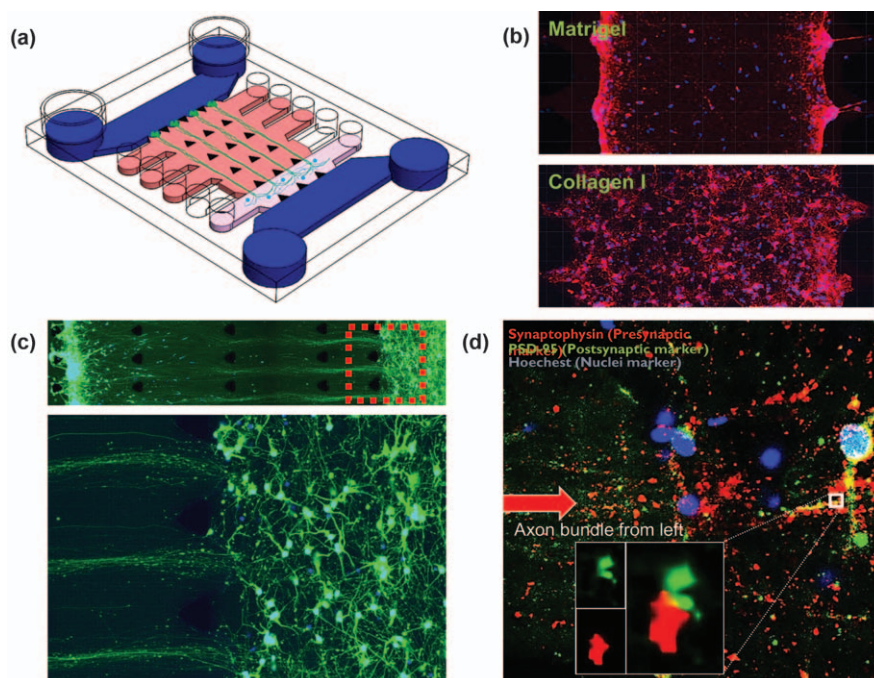


Figure 8.3 (a) Schematic of *in vitro* 3D neural circuit microfluidic platform. (b) Immunostaining images of hydrogel type in microfluidic platform. In Matrigel, it is difficult for the antibody to permeate, but in collagen, the antibody penetrates into the hydrogel. (c) Image of the axon bundle entering the post-synaptic neuron group. (d) Immunostaining of synapse of a functional neural circuit.

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8.3 Reconstruction of 3D BBB in Microfluidic Device

8.3.1 Introduction

Unlike the capillaries in the body, the capillaries presented in the brain are in contact with astrocytes. More specifically, astrocytic endfeet are anchored on the surface of capillaries. Therefore, these capillaries show distinctive features which cannot be seen in the blood vessels of other tissues. It is known as the blood–brain barrier (BBB). These structural features of the BBB lead to a specific function—that the permeability of capillaries is extremely low.¹² Due to this low permeability, substances in the blood cannot be transferred to the brain tissue. Therefore, the BBB can protect brain tissue from toxic substances, but conversely restricts drug delivery to brain tissue.

8.3.2 Methods for *In Vitro* 3D BBB Platform

8.3.2.1 Microfluidic Platform Fabrication

In the method of 3D BBB formation, the process of device fabrication, primary neural cell preparation, and immunostaining are the same as previously described in the section of *in vitro* 3D neural circuit platform.

8.3.2.2 Cell Plating for Vasculogenesis in the Microfluidic Platform

A fibrinogen solution was made by dissolving bovine fibrinogen (10 mg ml⁻¹, F 8630, Sigma-Aldrich) in Dulbecco's phosphate-buffered saline (DPBS, Hyclone) and filter-sterilized (0.22 μm pore). Then, this solution was mixed with aprotinin (0.15 U ml⁻¹, Sigma-Aldrich). Human umbilical vein endothelial cells (HUVECs) and Lung fibroblasts (LFs), which were detached from the cell culture dishes by treating with 0.25% Trypsin-EDTA (Hyclone), were centrifuged and suspended at a concentration of 6.7 million cells ml⁻¹ in EGM-2 medium. The cell suspensions are mixed with the fibrinogen solution at a ratio of 3 : 1 to yield a final concentration of both HUVECs and LFs as 5 million cells ml⁻¹. The mixtures with thrombin (0.5 U ml⁻¹, T4648, Sigma-Aldrich) were injected into the center hydrogel micro-channel and side micro-hydrogel channel. After 5 minutes at room temperature, the gel mixtures formed structures and the upper reservoirs in each device were filled with culture medium (EGM-2) which was aspirated gently at the lower reservoirs to make the hydrophobic medium micro-channel. Following even filling of the rest of the reservoirs with the medium, the devices were incubated at 37 °C and 5% CO₂. The culture medium was changed to fresh EGM-2 culture medium every 48 hours.

8.3.2.3 Area of Vascular Network and Astrocytes Measurement

The cross-sections of the vascular network and astrocytes were obtained by a confocal microscope (Olympus FV1000). Images were analyzed by ImageJ. To quantify the area of vascular network, Z-projections of the 3D stacks were obtained and then each image was masked from the backgrounds before measurement.

8.3.2.4 Permeability Coefficient Measurement

A fluorescence image of FITC-dextran diffusing across vascular network was analyzed to calculate the permeability coefficient. After removing all media from medium reservoirs, 50 μ l of 20 kDa FITC-dextran solution was passed into the media channel associated with the vascular network. 70 kDa FITC-dextran solution was treated the same way. An inverted epifluorescence microscope (Olympus IX81) was used for observation of the permeability of the vascular network. All images were taken in 25 second intervals and the fluorescence changes cross vascular network were analyzed.

8.3.3 Results and Discussion of *In Vitro* 3D BBB Platform

8.3.3.1 Formation of BBB by Co-culture of Neural Cell and Endothelial Cell

An important feature of the *in vitro* 3D BBB is intact co-culture of vascular networks and neural cells. First of all, the vascular networks should be perfusable. It is well known that when the endothelial cells and fibroblasts are mixed with fibrin gel and cultured separately in the microfluidic platform, a perfusable vascular network is formed towards both media channels similar to the vasculogenesis process.¹⁶ In this method, fibroblasts are usually plated over both media channels of the endothelial cell channel. However, for perfusable vascular networks towards only one media channel, fibroblasts were also plated on only one side. Although the vascular networks begin to be formed in the endothelial cell channel after 3 days, the lumen of the vascular network is not yet perfusably connected to the media channel. At this time, the cortical neural cells obtained from the rat embryo were attached to the walls of the endothelial cell channels on the opposite side of fibroblasts (see Figure 8.4a). After 3 days, the lumen of the vascular network was opened towards the side of the fibroblast, forming a perfusable vascular network. Also, the migrated astrocytes were in contact with the vascular network (see Figure 8.4b). The migration of astrocytes usually stops when they encounter a vascular network. Observation of this region in 3D by confocal microscopy confirmed that astrocytes and vascular networks made a number of direct contacts (see Figure 8.4c).

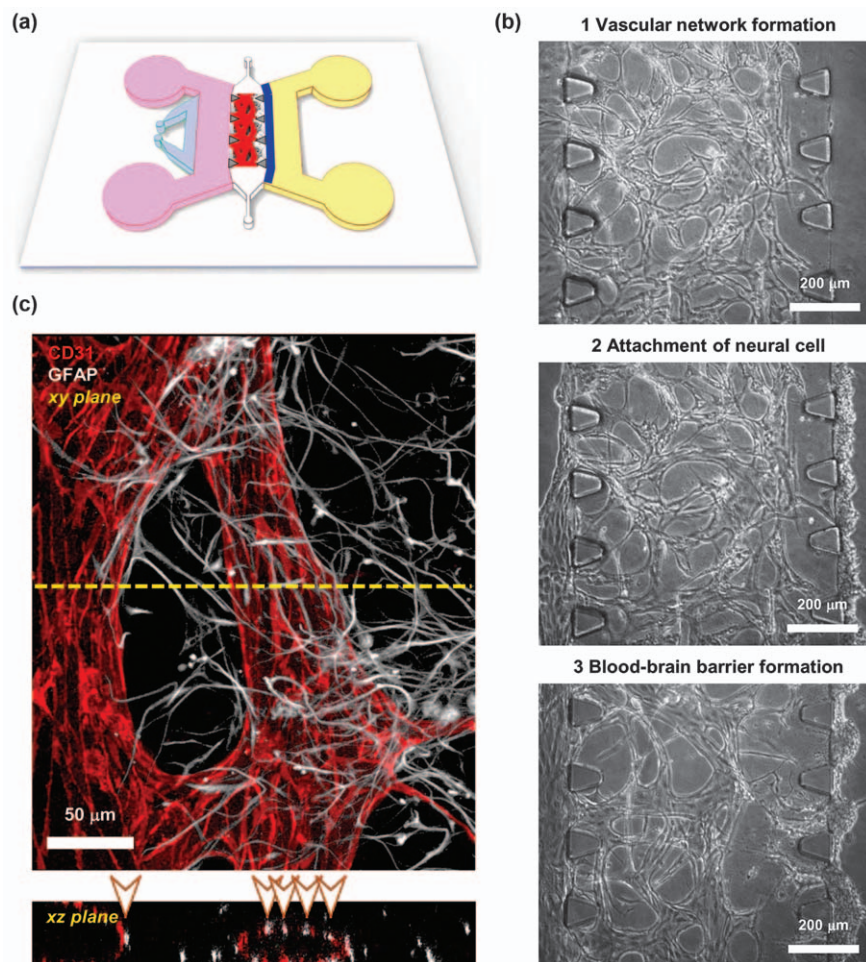


Figure 8.4 (a) Schematic of *in vitro* BBB microfluidic platform. (b) The process of BBB formation in the microfluidic platform. (c) Confocal image of the direct vascular network-astrocyte interface. Reproduced from ref. 15, <https://doi.org/10.1038/s41598-017-07416-0>, under the terms of the CC BY 4.0 license, <https://creativecommons.org/licenses/by/4.0/>.

8.3.3.2 Difference in Morphology and Function of BBB Depending on Media Composition Inside and Outside of Vascular Network

The *in vitro* 3D BBB microfluidic platform has perfusable vascular networks towards only one media channel. Furthermore, in this platform, it is possible to inject different media or materials inside and outside of the vascular network. The media channel associated with the vascular network is called

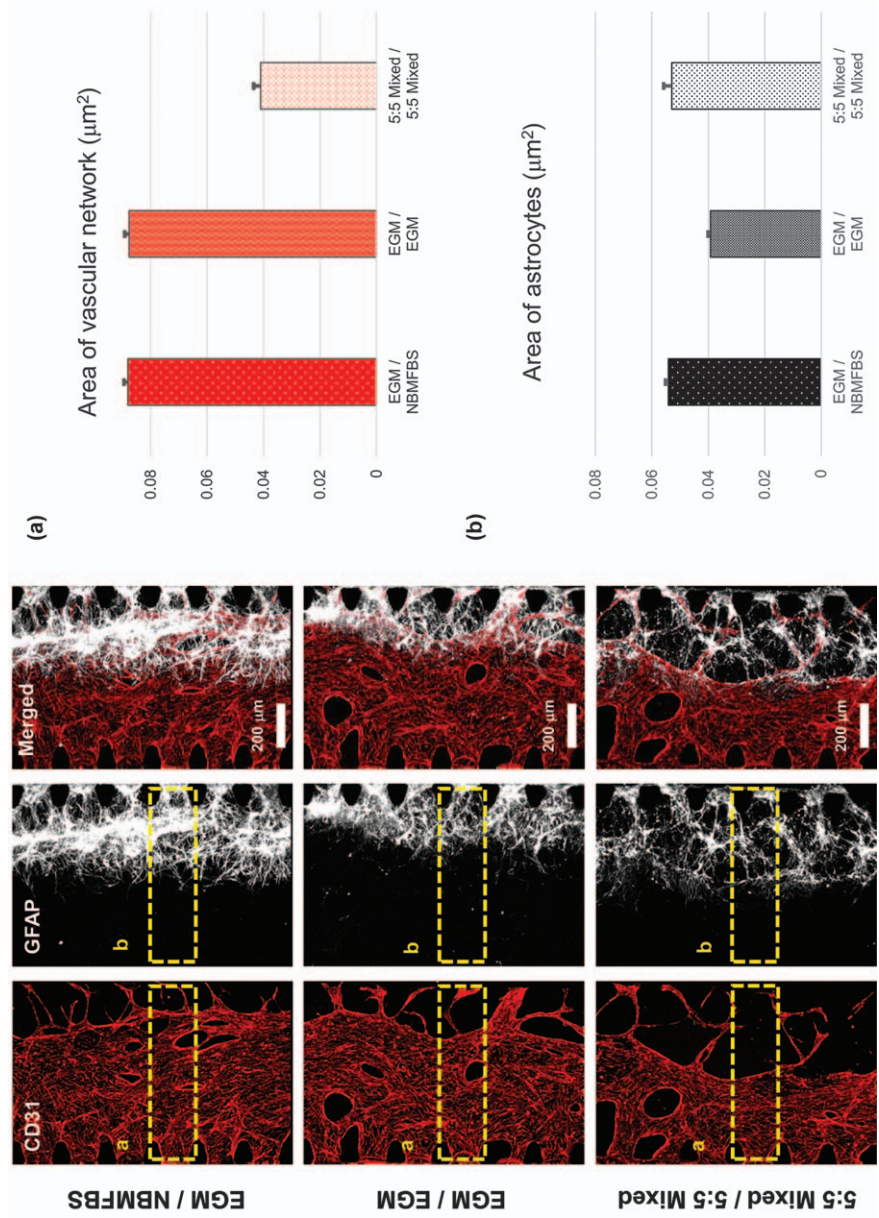


Table 8.1 Results of permeability experiments with 20 kDa and 70 kDa FITC-dextran. All data show mean $\pm$ SEM.

Model	20 kDa (10^{-6} cm s $^{-1}$)	70 kDa (10^{-6} cm s $^{-1}$)
EGM/none	1.85 $\pm$ 0.20	1.39 $\pm$ 0.19
EGM/EGM	0.65 $\pm$ 0.08	0.60 $\pm$ 0.12
EGM/NBMFBS	0.45 $\pm$ 0.11	0.36 $\pm$ 0.05
<i>In vivo</i> (ref. 18)	0.24	0.15

the vascular channel, VC, and the opposite channel is called the neural channel, NC. The types of media in VC and NC are as follows (annotated in VC/NC supplied channels): NBMFBS/EGM, EGM/EGM, 5:5 mixed/5:5 mixed. NBMFBS refers to neurobasal medium supplemented with FBS, and EGM is the abbreviation for endothelial cell growth medium. As a result, the area of the vascular network is maintained regardless of the media of the NC when EGM is present in the VC. On the other hand, if there is no intact EGM in the VC, the area of the vascular network is reduced rather than maintained. In other words, for the integrity of the vascular network, the EGM must be supplied to the inside of the vascular network through VC. On the other hand, there is a difference in the overlapping area between the vascular network and astrocyte depending on the kind of media supplied to the NC. By supplying EGM to VC, the area overlapping with astrocyte is larger when NBMFBS is present than when EGM is present in NC with constant vascular network area (see Figure 8.5).

In addition, the permeability test of the vascular network using FITC-dextran showed lower permeability when co-incubated with neural cells than with single-vascular network culture and lower permeability under NBMFBS/EGM condition than EGM/EGM condition (see Table 8.1). This is consistent with previously known results that as the direct contact between blood vessels and astrocytes increases, the permeability decreases.¹⁷

8.4 Conclusion

The microfluidic platform has been established as an *in vitro* model of brain tissue in two respects. First, regulating the cross-linking structures of the ECM components in Matrigel was possible in the microfluidic platform.

Figure 8.5 Comparison of vascular network and astrocyte areas in experimental medium co-culture conditions. CD31 stained vascular networks shown in red, GFAP stained astrocytes shown in white for all three experimental medium compositions (annotated in VC/NC supplied channels): EGM/NBMFBS; EGM/EGM; 5:5 mix of NBMFBS:EGM for both channels. (a) Average area occupied by the vascular network between a pair of parallel micro-posts. (b) Average area occupied by astrocytes between a pair of parallel micro-posts. Reproduced from ref. 15, <https://doi.org/10.1038/s41598-017-07416-0>, under the terms of the CC BY 4.0 license, <https://creativecommons.org/licenses/by/4.0/>.

Matrigel injected into the microfluidic platform showed a cross-linking density distribution due to the application of hydrostatic pressure during the gelation process. The axons were fasciculated along the sparse part of the density pattern of the deformed Matrigel. In addition, by adding a post-synaptic neuron group, the axon bundle was defasciculated and synapse was observed in the region, so that a 3D neural circuit reproduced *in vitro*.

Second, using a microfluidic platform, it was possible to make different environments inside and outside the vascular network. Asymmetric plating of the fibroblast and neural cells with respect to the vascular network contributed to the asymmetric formation of the vascular network. The vascular network formed the suitable environment for the blood vessel cell inside the lumen and for the neural cell outside the lumen, respectively. As a result, it was possible to recapitulate *in vitro* the structural and functional characteristics of the BBB *i.e.* a number of direct contacts between the vascular network and the astrocytes with low permeability.

This *in vivo*-like *in vitro* brain model is being approached from multiple perspectives as much as the complex brain. In the case of neural circuits, additional co-cultures may be used as a tool to replicate more complex neural systems such as myelination or neuromuscular junctions. In addition, the BBB may be used as a neurovascular model more similar to *in vivo* brain tissue through additional co-cultivation with pericyte or microglia. Neural circuit and the blood-brain barrier models developed on the microfluidic platform can lead to improvements in neuroscience and neuropharmacology.

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CHAPTER 9

3D Tissue Modelling of Skeletal Muscle Tissue

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9.1 Introduction

Skeletal muscle tissue constitutes approximately 40–45% of the overall weight of an adult human¹ and is responsible for voluntary movement and locomotion, as well as for sustaining proper posture. Therefore, deterioration of skeletal muscles leads to physical dysfunction and serious decrease in life comfort. Moreover, in the case of physical workers, soldiers or professional athletes, it might lead to dramatic changes in life-style, disability pension and further increase in the socioeconomic costs.

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Muscle damage can arise due to acquired or congenital conditions. The former includes muscle contusions, strains, lacerations and traumas which occur during accidents, sport activities, surgical interventions or even are induced by drugs (e.g. statin-related myopathy occurs as a side-effect in patients prescribed statin).² The latter include dystrophies among others.³

To a certain extent, muscle tissue exhibits an endogenous ability to regenerate. The self-regeneration occurs through activation of satellite cells.⁴ However, in the case of severe injury, the intrinsic mechanism of muscle repair becomes insufficient and an external intervention is required. Current treatment strategies are based on autologous muscle transplant in various forms. The most common include engraftment of healthy, vascularized and innervated muscle tissue taken from a site adjacent to the injury (so-called muscle flap) or taken from other remote sites (free functional muscle transfer).¹ However, both strategies suffer from disadvantages typical for autograft procedures, namely donor site morbidity and extended rehabilitation.

In this context, muscle tissue engineering (TE) emerges as a promising alternative. TE combines engineering and life sciences to develop “*biological substitutes that restore, maintain or improve tissue function*” by using infusions of cell suspensions, tissue-inducing substances or transplantation of cell-laden matrices.⁵ In the case of muscle TE, three approaches can be distinguished. The first one, namely *in vivo* TE, is based on intramuscular, intravenous or intra-arterial injection of a cell suspension.³ The cells can be also transplanted within artificial matrices, so-called scaffolds. However, the therapeutic outcome is rather poor due to low cell survival caused by the activity of immune cells present at the injured site. Another strategy (*in vitro* TE) involves the culturing of cell seeded scaffolds in bioreactors in order to obtain functional tissue analogues.⁶ In this case, proper vascularization, innervation, high myofibers packing and generation of physiologically-relevant contractile forces still have to be addressed. The last approach, namely *in situ* TE, seems to be the most promising as the graft is generated *in vivo* using biomaterials that deliver bioactive compounds and provide contact-guidance cues in order to recruit and activate endogenous stem cells.¹ Moreover, paracrine cell signaling can also be delivered within the biomaterials in an immune-protected manner.

In this chapter, we aim to give a comprehensive overview of regeneration and engineering of skeletal muscle tissue. Firstly, we provide description of the structure and functions of skeletal muscle tissue, followed by description of cell sources with potential for repair of muscle injuries and dysfunctions. Subsequently, we present natural and synthetic biomaterials, as well as techniques currently used to process them into TE scaffolds. We also discuss the importance of mechanical and electrical stimulation and their effect on differentiation and maturation of cells during *in vitro* culture. We will present the reported results of *in vivo* studies and provide a summary and outlook on future development in the field of muscle TE.

9.2 The Structure and Functions of Skeletal Muscle Tissue

The skeletal muscle tissue is responsible for the voluntary movement of bone segments and locomotion. This striated muscle tissue is composed of a set of muscle fiber elements with cylindrical irregular shape, deriving from the fusion of mononuclear cells, the myoblasts, that during embryogenesis fuse to form polynucleated syncytia, the so-called muscle fibers or myofibers.

The muscle fibers are associated in bundles and are held together by connective tissue. In particular, each muscle is formed by an outer connective tissue called the epimysium, which is continuous with the tendons inserted into the bones, guaranteeing the muscle functionality. A network of interstitial connective tissue septa originates from the epimysium, called the perimysium, that divides the muscle into compartments and surrounds individual bundles of muscle fibers, called fascicles. From the perimysium, in turn, springs up a system of thin and delicate septa of connective tissue called the endomysium, which envelops the individual muscle fibers (Figure 9.1), providing support and protection, allowing them to withstand the forces of contraction. This covering, moreover, supplies pathways for the passage of nerves and blood vessels that branch out in an extensive network of capillaries that surrounds each muscle fiber, ensuring the vitality and functionality of muscle tissue.⁷

The polynucleated muscle fiber is defined by the sarcolemma, the complex of plasma membrane and glycoproteins that envelops the sarcoplasm, the equivalent of the cytoplasm. Disseminated within the muscle fiber can be found numerous nuclei, arranged peripherally and surrounded by Golgi cisternae, glycogen particles and lipid droplets, a large number of mitochondria, essential given the great energy demand of these specialized cells during contraction, and sarcoplasmic reticulum, a specialized smooth endoplasmic reticulum with a complex system of membranes that surround the myofibrils playing a key role in the physiological muscle contraction process by calcium releasing.⁷

Myofibrils represent the contractile elements of muscle fiber and occupy most of the sarcoplasmic volume. They are cylindrical structure of 1–2 μm in diameter that extend longitudinally along the entire length of the muscle fiber, aligned to each other, giving rise to the characteristic pattern of light and dark bands visible in the longitudinal histological sections of skeletal striated muscle.⁸ This typical pattern depends by the organization and accumulation of thick, intermediate and thin myofilaments inside the myofibril. In particular, the light bands are called A bands; within them, a clearer area called the H band is present, which in turn is cut in half by the line M. The dark areas are called I bands and are cut in half by the Z disk. The contractile structural unit of the myofibril is the sarcomere, the area comprised between two Z disks measuring 2.5 μm in length.⁹

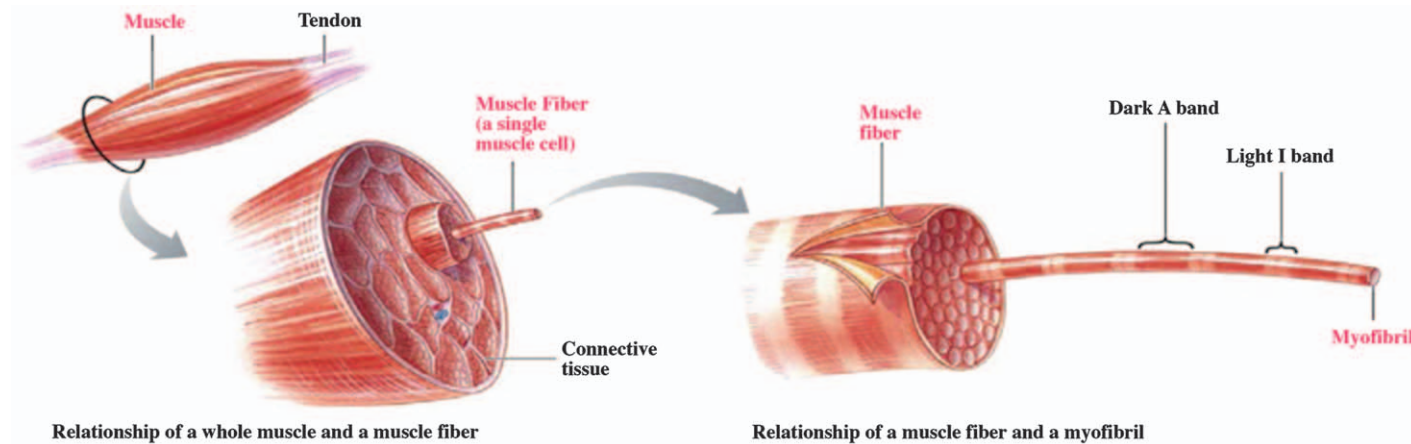


Figure 9.1 Structural organization of skeletal muscle at different scale levels.
Adapted from ref. 94 with permission from Springer Nature, Copyright 2014.

The sarcomere is mainly composed of two types of myofilaments: the thin myofilaments formed by F-actin, troponin and tropomyosin regulative proteins, which are inserted on the Z line to form the I band; and the thick myofilaments, consisting of bundles of myosin molecules that form A band together with thin myofilaments. The H band, the central region of the A band which possesses only thick myofilaments, is cut in half by the M line, mainly consisting of myomesin and M protein, with the function of maintaining the filaments in the correct position. The Z disk is the anchorage site of the actin myofilaments and is reinforced by non-contractile proteins such as α -actinin, titin and nebulin, which bind to each other and with myofilaments. In addition, desmin intermediate filaments are inserted on the Z disk, and along with proteins located near the sarcolemma such as dystrophin and other glycoprotein complexes forms a scaffold, anchoring the sarcomere contractile apparatus to the sarcolemma of the muscle fiber, ensuring its morpho functional integrity (see Figure 9.2).^{7,9}

During muscle contraction, the sliding of the thin myofilaments on thick ones occurs. Following contractile stimulation, myosin molecules initiate various cycles of binding, hydrolysis and release of ATP; at each cycle the myosin heads stick to the actin molecules of thin myofilaments, bend back making the actin filaments slide, and then detach and reattach upstream.⁸ As result, there is the contraction of the sarcomere with the approaching of the Z discs. After stimulation the myosin molecules detach completely and the sarcomere relaxes, returning to its starting length (see Figure 9.2).⁸

Muscle contraction is activated by a stimulus transmitted through a motor neuron axon to the neuromuscular junction, a particular kind of synapse; the presynaptic buttons release the neurotransmitter acetylcholine that binds its receptors located on the sarcolemma of the muscle fiber, triggering a potential action that propagates in a few milliseconds to a system of membranous tubes called transverse tubules (T tubules). These T tubules originate from the sarcolemma and then penetrate deep within the muscle fiber, going to wrap each myofibril. So the electrical signal is transmitted by the T tubules to the membrane system of the sarcoplasmic reticulum, which envelope the myofibrils. As a result, the high concentration of Ca^{2+} ions contained inside the sarcoplasmic reticulum is discharged into the sarcoplasm, due to the opening of voltage-dependent ion channels present on its membrane.¹ The increase in cytosolic calcium levels acts on the protein complex troponin C-tropomyosin present on myofibrils: in particular the calcium-sensing troponin C protein subunit in presence of Ca^{2+} ions undergoes to a conformational change that affects the tropomyosin protein associated with its end. Tropomyosin is a protein that binds and blocks seven actin monomers, preventing myosin from interacting with them but, following the conformational change of troponin C, it detaches from the actin monomers allowing to the myosin molecules to bind to actin filaments and start the contraction. In the opposite way, when the nervous stimulus stops, the cytosolic concentration of Ca^{2+} ions quickly returns to the basic level, resetting the system to the rest condition, with tropomyosin bound to the actin filaments.⁸

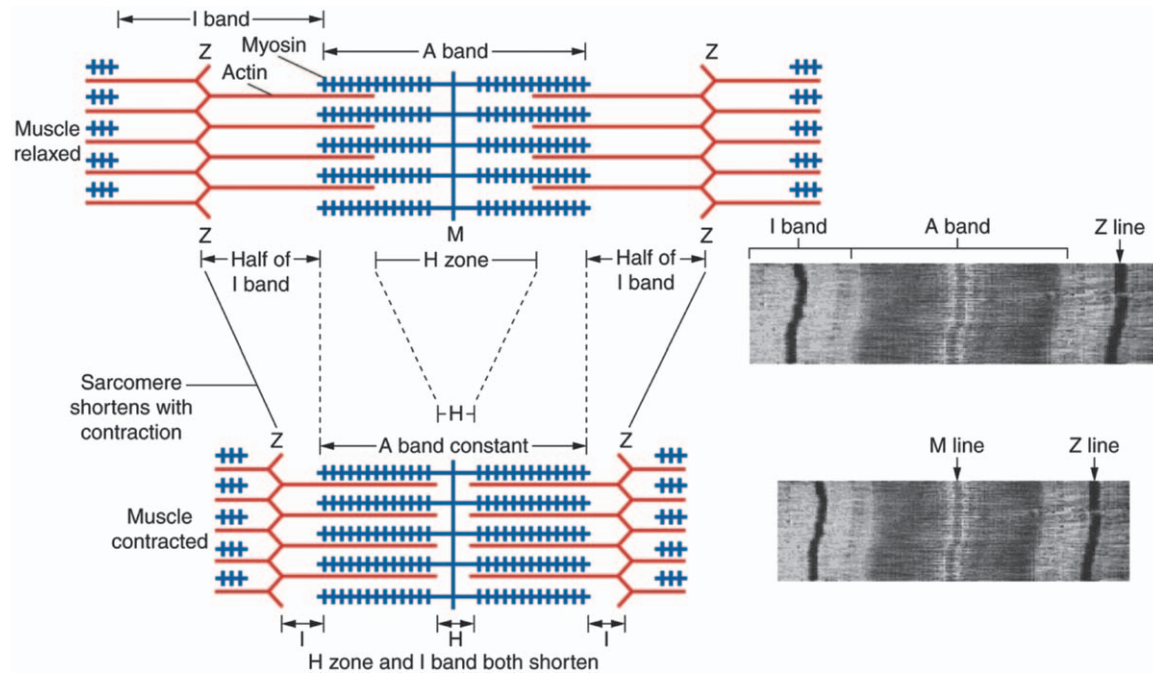


Figure 9.2 Structure of sarcomere: organization of the thin filaments (actin) and thick filaments (myosin) of sarcomere in relaxed (top) and contracted (bottom) state by schematic illustration (left) and electron microscope images (right). Reproduced from <https://oli.cmu.edu/jcourse/workbook/activity/page?context=df3c7ca80a0001dc27c9db0c6a565fd4> under the terms of the CC BY 3.0 license, <https://creativecommons.org/licenses/by/3.0/>.

9.3 Skeletal Muscle Regeneration

Mature skeletal muscle is composed by several bundles of myofibers, polynucleated syncytia, formed upon myogenic precursor cell, namely myoblasts, fusion. Adult skeletal muscle is a highly dynamic tissue able to self-renew and repair itself in response to increased workload, stress conditions or damage. Muscle regeneration is a fundamental homeostatic process orchestrated by different myogenic progenitor or stem cell populations guaranteeing the maintenance of muscle structure and function. This process can be schematized in four time-dependent phases. The first step is the necrosis of muscle fibers that activates a temporary muscle inflammation, necessary to remove necrotic cellular debris. The inflammation phase is followed by a regeneration phase in which stem cell populations replace damaged myofibers. The last phase consists of the remodeling of ECM and angiogenesis (Figure 9.3).¹⁰ Myogenic stem cells are cell populations able to differentiate into skeletal muscle fibers, and some of them can populate the muscle stem cell niche, thus contributing directly to regenerative processes. In pathological conditions or during ageing, the complete regenerative program can be precluded. In fact, it is demonstrated that the myogenic stem cell population compartments decrease with age in rats, mice and humans, and that the relative abundance of the different cell populations may change according to the strain, age and muscle type.¹¹

9.3.1 Cell Sources

In most adult mature tissue, there are quiescent progenitor (stem) cells appointed for tissue homeostasis maintenance which, in response to a damage, injury or growth demand, can take action undergoing proliferation and differentiation. Likewise, also skeletal muscle tissue (see Figure 9.4) presents different progenitor cells characterized by specific gene expression profile,¹² proliferation kinetics¹³ and molecular regulation.¹⁴

9.3.2 Satellite Cells

Satellite cells (SCs) are considered the most important myogenic progenitor cells in the muscle.¹⁵ Their description dates back to 1961 when Mauro for the first time described this cell population residing beneath the basal lamina above the sarcolemma in amphibians.¹⁶ Since then SCs are characterized by this peculiar localization (see Figure 9.5).¹⁷ The amount of SCs resident in skeletal muscle tissue decreases during a lifetime: in fact, during postnatal growth 30% of the sub-laminal nuclei decrease up to 1–5% in adult life, in relation with the formation of muscle fibers by the fusion of the same cells.¹⁸ SC reacts upon a damage stimuli, switching from quiescent to activated cells, proliferating and fusing with regenerating myofibers or forming polynucleated syncytia and giving rise to new myofibers for muscular tissue regeneration.¹⁹ Activation signals are well characterized

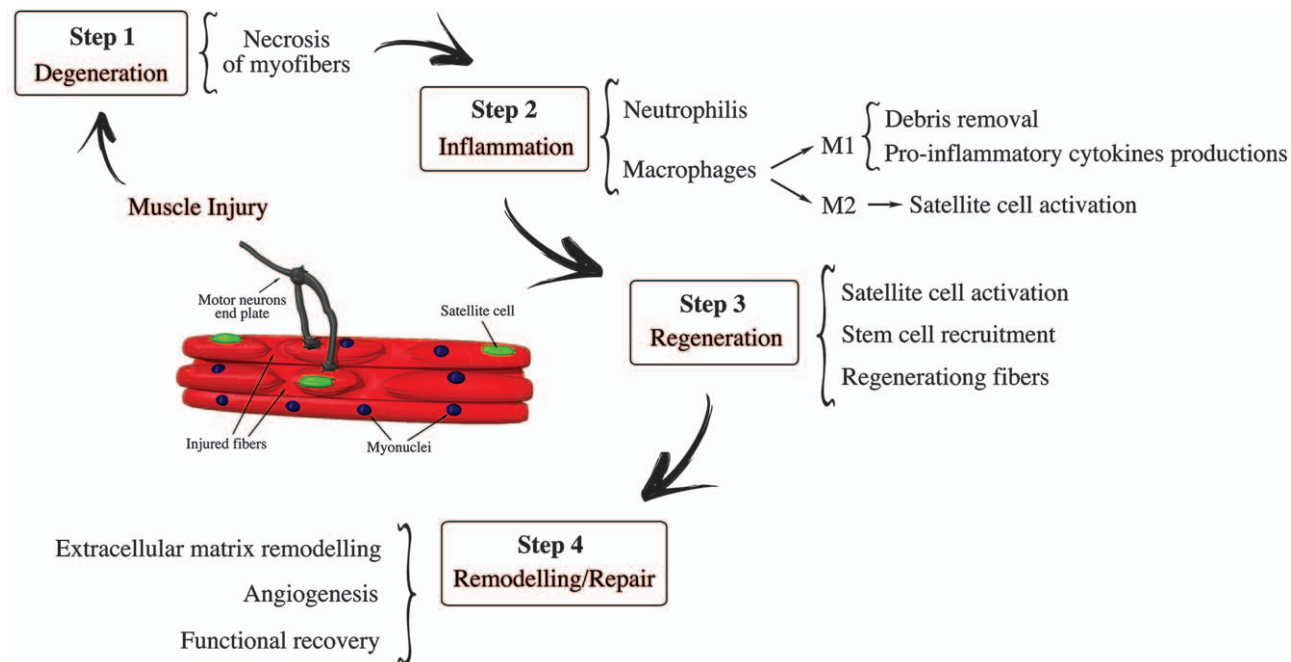


Figure 9.3 Schematic representation of 4 phases of skeletal muscle regeneration. During muscle regeneration 4 different phases can be distinguished: degeneration (Step 1), inflammation (Step 2), regeneration (Step 3) and repair (Step 4).

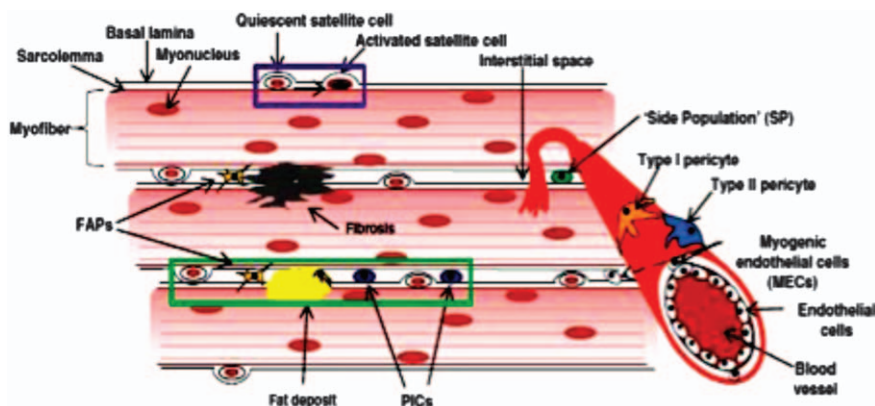


Figure 9.4 Schematic representation of skeletal muscle mononuclear cell populations. FAPs; PICs – PW1(+) interstitial cell. Reproduced from ref. 95 with permission from Springer Nature, Copyright 2015.

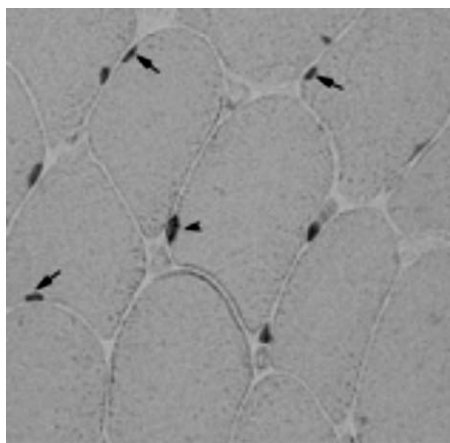


Figure 9.5 Muscle SC localization. Identification of satellite and myonuclei cells. Cross section of muscle stained with an antibody for CD56 (black) and counterstained with haematoxylin. The myonuclei are grey, and the SCs are surrounded by a brown outline. Arrowheads indicate SCs while arrows show the myonuclei. Reproduced with permission from ref. 96, Copyright 2004 The Journal of Physiology and The Physiological Society.

thanks to well-defined SCs molecular signatures—Pax-7 expression being the specific marker typifying quiescent SCs besides other markers: M-chaderin, c-Met (HGF receptor), Syndecan-4, CD34 (Cluster of Differentiation 34), $\alpha 7$ -integrin e CXCR4 (CXC Chemokine Receptor 4).^{13,15}

The first event marking SCs activation is p38 (MAP kinase) phosphorylation followed by Myf5, MyoD and Myogenin expression (Muscle Regulatory

Factors) leading to myoblast differentiation and then to fusion and myofibers formation and reconstruction.^{20,21}

9.3.3 Pericytes

Along with SCs, in a different cell niche represented by the perivascular compartment (around small and medium blood vessel), a mononuclear population—namely pericytes—is localized. They were initially described as Rouget cells after Charles-Marie Benjamin Rouget, until 1923 when Zimmermann coined the term pericyte.²² These cells have been described and isolated from several tissues. Being periendothelial cells residing within microvasculature, they regulate numerous functions such as angiogenesis and vessel growth, permeability and contractility enfolding abluminal surface of capillaries, pre-capillary arterioles, post-capillary venules, and veins with fingerlike projection from the round cell body.²³ Skeletal muscle pericytes (MPs) showed multipotential capability, *i.e.* being able to differentiate in different mesodermal cell lineages such as adipocyte, smooth muscle and skeletal muscle in relation to cell niche and differentiation stimuli.²⁴ MPs play several functions in skeletal muscle tissue including modulating angiogenesis and blood flow as well as stabilization of the SC pool promoting quiescence through Ang1 (Angiotensin 1) and IGF1 (Insulin like Growth Factor 1) dependent activation.²⁵ Moreover, MPs are also considered an important source of myogenic progenitors distinct from SCs.²⁶ While SCs hold a discriminatory specific marker (Pax7) MPs do not, hence different markers are utilized to identify univocally pericytes. The most used and accredited are: Alkaline Phosphatase (ALP), Platelet-Derived Growth Factor Receptor beta (PDGFR β), NG2 (Neural/Glial antigen 2), CD13, alpha smooth muscle actin (α SMA), nestin and CD146.²⁷ Recent studies demonstrated that ALP⁺/MPs (see Figure 9.6) are able to undergo SCs differentiation expressing Pax7. Moreover, patients affected by muscle dystrophy revealed an increased amount of ALP⁺/MPs on muscle biopsies during tissue regeneration.²⁸

9.3.4 Fibro-adipogenic Progenitors

Fibro-adipogenic progenitors (FAPs) are mesenchymal progenitor cells located in the skeletal muscle interstice playing a key role during muscle regeneration by positively regulating SCs' differentiation. They are bipotent cell populations with a potential to differentiate into both adipocytes and fibroblasts. FAPs are characterized by the expression of cell surface markers, Stem cell antigen 1 (Sca-1), common interstitial marker, Platelet-Derived Growth Factor Receptor alpha (PDGFR α) and CD34, and by the absence of SC specific markers such as α 7-integrin and SM/C-2.6, and markers specific for hematopoietic and endothelial cells, CD45 and CD31, respectively.²⁹ FAPs, upon muscle damage, activate presenting a proliferation peak around 3–4 days after injury, releasing pro-myogenic paracrine factors such as IL-6 and IGF-1 that promote satellite cell differentiation.³⁰ After expansion, FAPs

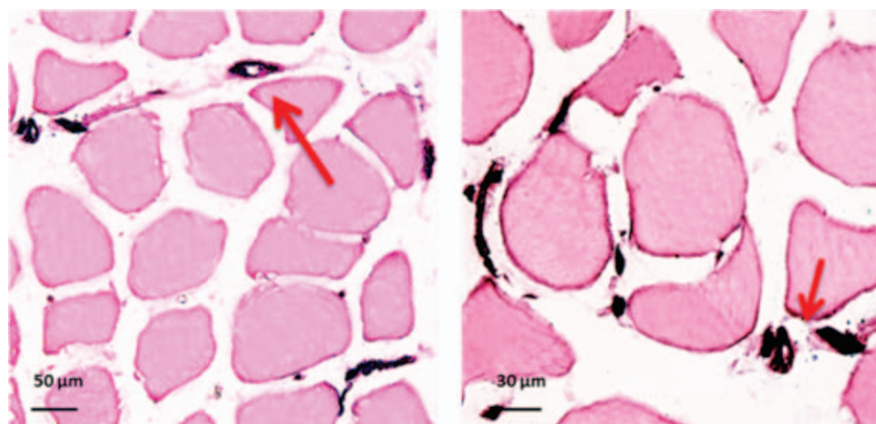


Figure 9.6 Mouse skeletal muscle transversal section labelled with AP staining and Eosin revealing AP⁺/MPs (dark blue) closely associated to blood vessels (red arrows), myofibers are labelled in pink.

undergo a rapid reduction in cell number, in order to remove excessive cells generated during the proliferation phase, and return to the initial quiescent state around 7–9 days post injury.²⁹ In addition, FAPs have been reported to be involved in debris removal from the injured muscle alongside macrophages.³¹ Nonetheless, in pathological conditions such as muscular dystrophies, instead of returning to the quiescent state, they take an opposite direction and choose to differentiate, becoming the main source of adipose and fibrous tissue accumulation.³²

9.4 Biomaterials

Biomaterial scaffolds aim to restore and/or replace the extracellular matrix (ECM) function, guiding and promoting the tissue regeneration. In this frame, 3D substrates composed of natural or synthetic materials as well as hybrid constructs have been investigated. Materials intended for this purpose should be biocompatible showing high surface area for cell adhesion, proliferation and matrix deposition. Cell guidance and control of cell differentiation are also very crucial aspects. Moreover, the possibility of cell, bioactive molecule or drug encapsulation and delivery through the biomaterial scaffolds is considered a big advantage to improve the tissue regeneration and induce vascularization.¹

The biomaterial scaffolds should possess adequate structural, mechanical, physical and biological properties matching as close as possible those of the targeted tissue in order to provide mechanical support and mimic the native tissue properties. The induction of myogenic cell differentiation and myotubes formation is a crucial point in the design of systems for muscle TE. Thus, several biomaterials with different geometries, shape and chemical properties have been designed and developed for muscle TE. Scaffolds

composed with an aligned fibrous structure are considered the best candidates to reproduce the anisotropic-oriented structure of the native muscle, potentially influencing myoblast alignment and fusion. Biofunctionality and stimulation of vascularization and innervation have also been addressed.³³

9.4.1 Decellularized Matrix

Decellularized extracellular matrices (dECMs) are engineered constructs that result from the decellularization process of organs or tissues. This process is based on the use of enzymatic physicochemical methods for removing cells and DNA from native ECM.³⁴ dECM is considered an ideal option for TE applications due to the presence of native ECM structure and composition. Moreover, the preserved matrix proteins and cytokines including collagen types I, III, IV, V, VI, VII, laminin, fibronectin, and glycosaminoglycans (GAGs) such as heparin, heparan sulfate, chondroitin sulfate and hyaluronic acid and growth factors on the construct surface play important roles in cell-material interaction as well as in cell response in terms of cell attachment, migration, proliferation and differentiation.³⁵

Porcine small intestine, dermis, porcine urinary bladder, pericardium and heart valves are the most common FDA-approved decellularized matrices.³⁶ In particular, bladder extracellular matrix was successfully used as a 3D scaffold for muscle TE.³⁷ The authors demonstrated that the construct possessed similar functionality compared to muscle physiologic tissue and was capable of active contraction and force generation.³⁸ Significant results were obtained in restoring the functionality of the muscle tissue in a volumetric muscle defect model *in vivo*.³⁹

Moreover, bioinks composed of decellularized muscle matrix were also developed. Such materials were loaded with cells and 3D printed, showing a successful reproduction of the anisotropic fibrous structure of the native tissue and cell orientation.⁴⁰

However, the use of decellularized ECMs have shown some disadvantages including low availability of donor tissues, donor site morbidity or reaction of the host immune system. Moreover, the decellularization process is generally not totally efficient and few DNA segments may be persistent on the scaffolds, while the aggressiveness of the process may change the mechanical, biochemical and structural properties of the matrix.³⁴

9.4.2 Natural-derived Biomaterials

Natural-derived materials have been recently considered in the fabrication of scaffolds for muscle TE due to their bioactive properties and the high availability of cell attachment sites. Mechanical and physical properties of the constructs can be easily tuned by regulating the polymer concentration. Moreover, this class of materials permits the incorporation of bioactive molecules as well as they can be chemically modified to add specific functional groups.⁴¹ Because of their unique properties, natural-based

biomaterials are most commonly exploited as hydrogel constructs. Hydrogels are considered excellent candidates for the fabrication of scaffolds for muscle regeneration due to their high water content, similarity to components present in the native tissue and minimal risk of inflammation. Moreover, hydrogels can conform to the defect geometry.⁴²

A variety of hydrogels have been tested as bioinks for 3D printing scaffold fabrication. During the printing process, the control of the mechanical forces applied on the hydrogel has been proven to promote cell orientation and alignment.⁴³

Collagen is the most common extracellular matrix component in the connective tissues, skin and bone. As a consequence, it is the most exploited natural material for TE applications because it properly reproduces the ECM composition. Collagen-based hydrogels have been tested in muscle engineering applications to stimulate the myogenic expression and the formation of myotubes. It has been demonstrated that myoblasts encapsulated into collagen hydrogels have the capability to migrate in the defect site and fuse with the native myoblast cells, accelerating the regeneration process.

Collagen-based 3D hydrogels, porous structures and coatings are the most common options used to promote cell adhesion, spreading, migration and proliferation. Moreover, the possibility of creating gel microgrooves resulted in reproduction of the bundle structures of the native muscle, improving cell alignment and myosin expression.⁴⁴

The microfibrillar nature of fibrin, a fibrinogen-derivate protein, made it an interesting candidate for the production of microthreads which can recapitulate the muscle cable-like structure. Fibrin microthreads were demonstrated to favor cell alignment without eliciting any inflammatory response. It has been demonstrated that fibrin exhibits an even higher myogenic potential compared to collagen, thus playing a fundamental role in myoblasts activation, proliferation and fusion.⁴⁵

Chitosan, a polysaccharide extracted from chitin, was also exploited for muscle TE. Its abundance in nature, similarity to GAGs structure (the second major component in ECM) and non-immunogenicity made it one of the best candidates for scaffold production. For instance, thanks to its simple processability, chitosan can be easily used to create aligned nanofibrous material and porous structure. In the first case, chitosan showed adequate mechanical properties and alignment of myoblasts seeded on top,⁴⁶ while porous chitosan scaffolds improved cell fusion and infiltration, allowing the formation of large myotubes.⁴⁷

However, it is important to underline some limitations of the natural based materials. First, their properties do not match perfectly the ECM morphology and structure, resulting in lacks of structural, chemical, biological, and mechanical complexity. In particular, their mechanical properties are considered weak in most of the cases and their reproducibly appear poor due to batch-to-batch variability. Moreover, the precise control of their properties is often complicated. Immunogenic risk is low but still exists and the processability in complex 3D shapes is still challenging.

9.4.3 Synthetic Materials

Synthetic polymers gained notable attention in the fabrication of scaffolds for TE due to their excellent mechanical, physical and structural properties which can be easily tuned controlling the synthetic process.⁴¹ These materials can be precisely tailored into engineered scaffolds with specific geometries and/or configuration and they can be produced in the form of mesh, foam or hydrogel. Electrospinning of polymers is the most utilized technique for the obtainment of aligned and oriented nanofibers thus mimicking the native ECM fibrous structure.⁴⁰ Moreover, the possibility of functionalizing the material structure by chemical modification and the incorporation of growth factors and bioactive molecules made synthetic biomaterials suitable for TE application.⁴⁸

The main biomaterials used for muscle TE are FDA-approved polyesters including poly(ϵ -caprolactone) (PCL), poly(lactic acid) (PLA), poly(glycolic acid) (PGA), and their copolymers. However, several polyurethanes have been also considered due to the efficient chemical functionalization which leads to a precise regulation of degradation rate, hydrophobicity and mechanical properties of the materials.⁴⁹

PCL is widely utilized because of its easy processability in terms of melting temperature (around 60 °C), great miscibility in many solvents and the possibility of blending with other polymers. It is reported that PCL has adequate mechanical properties and a slow degradation *in vivo* (a few months or years). For these reasons, it is mainly used for long-term muscle replacements.⁵⁰

Poly(lactic-co-glycolic acid) (PLGA) is a copolymer that has also been investigated for this application due to its good biocompatibility, high stiffness and nontoxicity of its degradation products. PLGA scaffolds have been fabricated in the form of electrospun mats in order to produce aligned fibrous constructs for myotubes elongation and orientation,⁵¹ as well as porous scaffolds to facilitate cell infiltration and vascularization.⁵²

However, it is necessary to point out some general disadvantages of synthetic polymers such as the lack of biological recognition sites for cell attachment as well as inflammatory response of the host body with possible formation of fibrotic tissue.

9.5 *In Vitro* Models for Skeletal Muscle Regeneration

Scaffolds are designed to mimic the native extracellular matrix (ECM), guide cell growth into defined geometries, and possess adequate surface properties, porosity and mechanical properties. They can also act as delivery vehicles for growth factors to enhance regeneration, guide nerve growth and induce vascularization. With these properties in mind, scaffolds for skeletal muscle must facilitate parallel alignment of muscle fibers as in the native tissue, enable myogenesis, and promote vascularization.⁵³

Meshes or grids of polymers with interconnected porosity and channels may promote cell alignment inside a 3D scaffold. The challenge is to then

design a geometry that mimics the natural orientation of skeletal muscle ECM, avoids the pitfalls of a dense matrix which could result in a necrotic or acellular core and yet maintains structural properties of a tensile and weight-bearing construct.

9.5.1 Electrospinning

In skeletal muscle TE, the most critical feature of mature muscle is the formation of aligned muscle fibers. Despite being known for decades, electrospinning has been extensively employed in TE only during the last 30 years to produce nanofibrous structures.⁵⁴ Attaining aligned fibers through electrospinning technique is challenging; however, various setups have been proposed. Most of these setups are based on mechanical rotation of the collecting mandrel,⁵⁰ electrical field manipulation⁵⁵ or on their combination. Fiber alignment can also be influenced by the composition of the electrospinning solution. For instance, McKeon-Fischer *et al.* reported that poly(3,4-ethylenedioxythiophene) (PEDOT) nanoparticles dispersed in a solution of poly(ϵ -caprolactone) allowed the formation of nanofibrous electrospun composite scaffolds on a rotating mandrel collector. As the PEDOT nanoparticle concentration was increased, the fiber alignment was significantly affected and shifted into random arrangement, while the conductive properties of the constructs were improved.⁵⁰

Another challenge in electrospinning is to attain significant sizeable scaffolds for the treatment of critical skeletal muscle defects. Thus, highly ordered self-assembly fibrous bundles were suggested by Lee *et al.*⁵⁶ A SPION (super paramagnetic iron oxide nanoparticles) loaded PLGA (poly(L-lactide-co-glycolide)) suspension was electrospun on a copper stick and seeded with myoblasts. After C2C12 myoblasts grew on the fibrous bundles, they fused together and differentiated into multinucleated myotubes. Moreover, to attain a substantial sized muscle, an external magnetic field was applied to induce self-assembly of the cell rods into a 3D cell-dense tissue with a highly oriented structure.

9.5.2 Bulk Hydrogels

Electrospinning is a well-established technique to obtain aligned nano-sized fibers. However, these scaffolds are not suitable for minimal invasive implantation. Furthermore, enlarging the scaffold thickness dramatically reduces the scaffold porosity, as well as nutrient diffusion and cell penetration through the scaffold. These drawbacks found with electrospun matrices can be overcome using a hydrogel scaffold. Hydrogel scaffolds are networks composed of hydrophilic polymers which can absorb huge amounts of water or biological fluids and swell readily without dissolving.⁵⁷ One of the greatest advantages of hydrogels is that cells can be encapsulated into the hydrogel network, and the overall system can be easily injected and cured *in situ*. Studies using hydrogels mimicking the elasticity of skeletal muscle have demonstrated that there is improved stem cell regeneration and

differentiation.⁵⁸ Salimath *et al.* used PEG-maleimide hydrogels for the encapsulation of C2C12 cells and showed that these hydrogels provided appropriate signals for the development and to a certain degree the self-alignment of myotubes, which represents the first step towards the emulation of native skeletal tissue.⁵⁹ Bulk hydrogel systems lead to an inadequate degree of cell fusion and limited aligned fiber formation. The disorganized and isotropic network structure of bulk hydrogels is the most important limitation for their applications in skeletal muscle TE. Thus a new class of injectable nanocomposite cell-laden hydrogels based on hydrazone cross-linked poly(oligoethylene glycol methacrylate) and magnetically-aligned cellulose nanocrystals (CNCs) (see Figure 9.7) were reported to promote skeletal muscle myoblasts differentiation into highly oriented myotubes.⁶⁰ Additionally, it is also worth noting that the mechanical stiffness of the hydrogels plays an important role in myoblast differentiation and myotube alignment. In a recent study, it was reported that the increased stiffness of gelatin methacrylate hydrogels, significantly decreased the ability of C2C12 to undergo rapid and efficient myogenesis.⁶¹

9.5.3 3D Printing

Bioprinting is an emerging TE technology that holds promise for fabricating skeletal muscle. Customized printers can deposit bioinks to create a variety of 3D shapes. The advantage of this technology is the ability to precisely position every element of a construct according to a predetermined 3D design. For instance, Kang *et al.* built a four-material printer which fabricated 3D scaffolds for bone, cartilage and skeletal muscle TE where synthetic polymers were printed as a supporting outer scaffold, with rows of bioinks printed within.⁶² Similarly, PCL supporting decellularized ECM bioinks were also printed in the desired spatial pattern to generate various types of 3D muscle constructs. In this case, it was observed that such constructs cultivated in myogenic conditions exhibited high viability, proliferation, myotube formation, and myogenic differentiation.⁶³

Recently a new strategy based on direct 3D bioprinting for the fabrication of artificial skeletal muscle tissue with functional morphologies was suggested by Costantini *et al.*⁶⁴ The proposed system was based on a micro-fluidic printing head coupled to a coaxial needle extruder for high-resolution 3D bioprinting of muscle precursor cells (C2C12) laden hydrogel fibers (Figure 9.8). Inspired by the native structural morphology of skeletal muscles, the spatial confinement of highly aligned muscle precursor cells into a compact 3D bioprinted hydrogel fiber resulted in a distinctive orientation of the arising myotubes, thus mimicking the natural muscle morphology and organization more closely.⁶⁴

9.6 Induction of Differentiation

Since the pioneer works of Vandenburg and colleagues at the beginning of the 90s reporting the first 3D model of skeletal muscle tissue mechanically

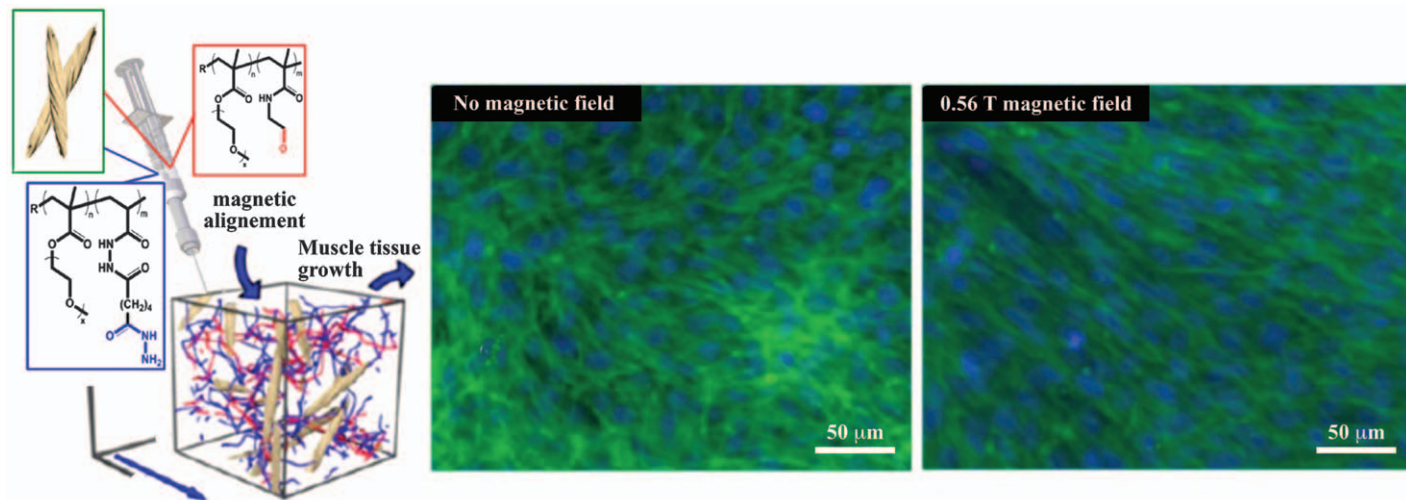


Figure 9.7 (Left) Nanocomposite hydrogels based on hydrazone cross-linked poly(oligoethylene glycol methacrylate) and magnetically-aligned cellulose nanocrystals (CNCs), and phalloidin/DAPI staining of C2C12 mouse myoblasts cultured on (middle) unaligned and (right) aligned hydrogels after 8 days of culture in differentiation media. Reproduced from ref. 60 with permission from the American Chemical Society, Copyright 2017.

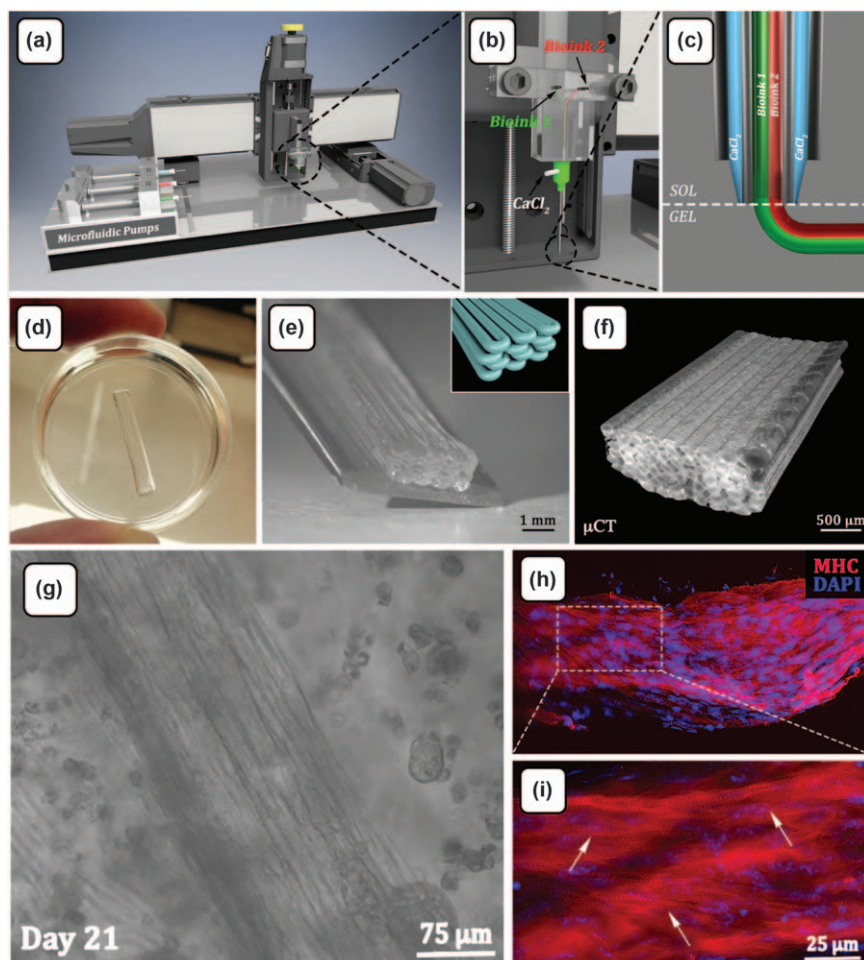


Figure 9.8 (a) Custom-built 3D printer equipped with fully programmable microfluidic pumps. (b) Microfluidic printing head coupled to (c) coaxial extruder. (d) Macrograph of a 3D bioprinted construct into a 35 mm Petri dish. (e) High magnification optical micrograph of the construct; a schematic representation of fiber arrangement is reported in the inset. (f) X-ray microtomographic scan of the sample in (d). (g–i) Day 16 showing a well-organized myostructure with the first evidence of sarcomere formation (white arrows). Reproduced from ref. 64 with permission from Elsevier, Copyright 2017.

stimulated,⁶⁵ researchers have developed and refined several approaches and protocols to induce the differentiation *in vitro* of skeletal muscle progenitors (Figure 9.9). Such approaches aim at recreating the *in vivo* conditions in which skeletal muscle tissue develops and functions so as to guide cell differentiation. In particular, muscle progenitors are generally exposed to two types of stimuli: mechanical and electrical. Each of these stimuli is

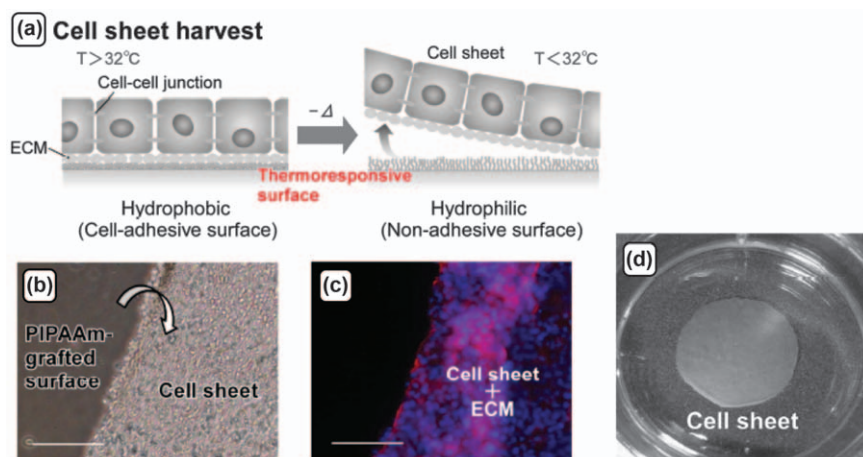


Figure 9.9 Cell sheet-based TE. (a) Schematic illustration of thermally induced cell sheet detachment with preservation of cell–cell junctions and associated ECM. Due to the surface alternation, a cell sheet can be harvested intact simply by lowering culture temperature. (b) Phase contrast and (c) fluorescence microscopic images of a cell sheet detaching from a PIPAAm-grafted surface. The associated ECM detached with the cell sheet from the surface, which is important for cell sheet transplantation. Fibronectin and cell nuclei were stained with red and blue, respectively. Scale bar: 100 μm . (d) Photograph of a cell sheet harvested from a thermoresponsive cell culture dish. The cell sheet shrunk two-dimensionally, but kept the original shape. Reproduced from ref. 97 with permission from John Wiley and Sons, Copyright 2015 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

provided to recapitulate a particular feature present *in vivo*. Mechanical stimulation, for instance, simulate both the active (controlled by motoneurons) and passive tension (unrelated to innervation) under which skeletal muscle tissue assembles, functions and regenerates from the embryonic to the mature state. Electrical stimulation is aimed at mimicking the action of the motoneurons found in native physiological environment.

So far, researchers have developed protocols in which such types of stimuli are provided individually⁶⁶ (single stimulus) or simultaneously⁶⁷ (multiple stimuli) to enhance the differentiation capacity of muscle progenitors.

9.6.1 Mechanical Stimulation

Among the two stimuli applicable to skeletal muscle progenitors, mechanical stimulation is undoubtedly the most studied with an abundant literature published. Mechanical stimulation can be provided in two different ways through static or dynamic systems. In static systems, cells are exposed to a constant tension while in dynamic systems the timing, duration and amplitude of induced mechanical stress (*i.e.* duty cycle) is varied on time, generally alternating periods of mechanical stimulation with periods of rest.

The first system reported for the mechanical stimulation of a 3D model containing skeletal muscle progenitors was done by Vandenburg and his colleagues in the mid-90s.⁶⁵ In this pioneer work, primary myoblasts were isolated from the forelimbs and hindlimbs of rat neonates and encapsulated into Matrigel-collagen solution (Figure 9.10a). Such constructs were then cultured within custom rectangular wells that had two stainless steel caps to allow the attachment of the 3D constructs, thus providing a static mechanical stimulation. The authors found that myoblasts rapidly differentiate forming aligned long-range myotubes that remained vital and functional over the studied period (3 weeks). Few years later, the same authors proposed an advanced system to provide an active mechanical stimulation to six 3D constructs simultaneously.⁶⁸ In this work, they encapsulated primary human cells within a blend of collagen and Matrigel and cultured them under dynamic conditions simulating forces associated with muscle organogenesis and exercise. It turned out that dynamic mechanical conditioning, when compared to static culture, increased mean myofiber diameter by 12%

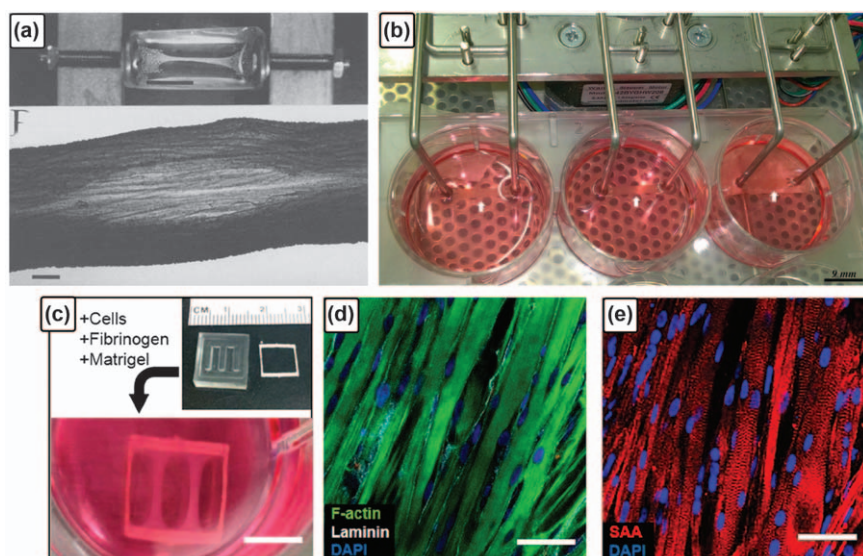
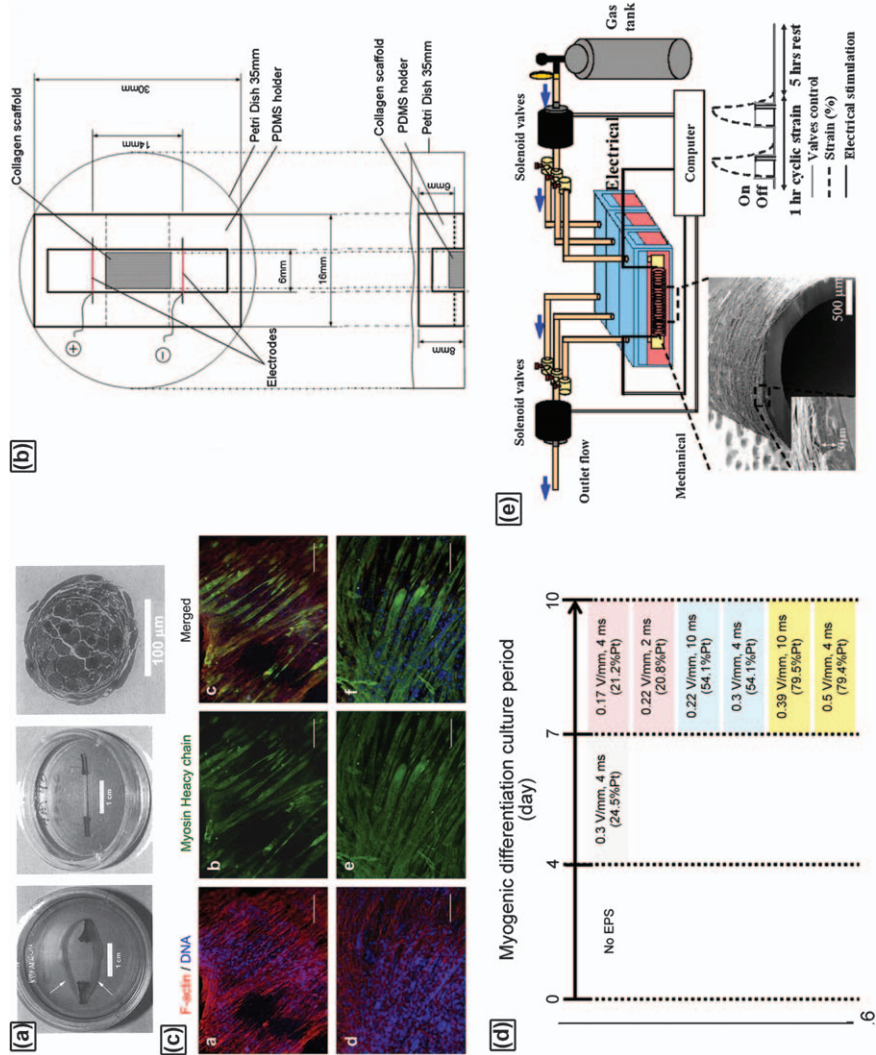


Figure 9.10 (a) (Top) First prototype capable of providing a static mechanical stimulation. (Below) Magnification of the skeletal muscle organoid composed of aligned myofibers.⁶⁵ (b) Active mechanical stimulator: the mechanical stress is provided by a stepper motor that moves stainless steel pins to which samples are attached.⁹⁸ (c–e) Human myogenic precursors embedded within fibrin/Matrigel matrix, casted into a PDMS mold and anchored to nylon frames to provide static mechanical stimulation. In (d) and (e) aligned myofibers positive for F-actin showing striated pattern of the contractile protein sarcomeric α -actinin (SAA).⁹⁹ Part a reproduced from ref. 65 with permission from Springer, Copyright 1997. Parts b, d and e reproduced from ref. 98, <https://doi.org/10.1111/jcmm.13186>, under the terms of the CC BY 4.0 license, <https://creativecommons.org/licenses/by/4.0/>, Copyright The Authors 2017.



and the area covered by myofibers by 40% after 8 days of mechanical stimulation.

In other work, Liao *et al.*⁶⁹ fabricated a custom cylindrical bioreactor trough which supplied an electromechanical stimulation to mouse skeletal myoblasts (C2C12) seeded on top of aligned or random electrospun mats (Figure 9.11e). Notably, they found that C2C12 cultured on aligned polyurethane fibers showed more pronounced elongation, better alignment, higher levels of transient receptor potential cation channel-1 (TRPC-1) expression, upregulation of contractile proteins (such as actinin and myosin) and higher percentage of striated myotubes compared to those cultured on random PU fibers and film. Similarly, Candiani *et al.*⁷⁰ – by employing a system similar to the one in Figure 9.10b – demonstrated that cyclic mechanical stimulation favors myosin heavy chain (MHC) accumulation in engineered skeletal muscle constructs composed of microfibrillar electrospun membranes seeded with C2C12. Interestingly, the authors first cultured the samples for 5 days in static conditions and then transferred the constructs into the dynamic bioreactor to mimic mouse development and growth. Western blot analysis showed that, at day 10, samples cultured in dynamic conditions exhibited 8 times higher accumulation of MHC with respect to samples cultured in static conditions.

To summarize, the main effects of mechanical stimulation – as static or dynamic – over 3D constructs loaded with skeletal muscle precursors consists of (i) the assembly of a more functional organization of the multinucleated myofibers with the formation of aligned bundles of fibers, (ii) more functional contractility, and (iii) more pronounced differentiation of cells.

9.6.2 Electrical Stimulation

Similar to mechanical stimulation, electrical stimulation is crucial to recapitulate the *in vivo* niche needed to fabricate functional skeletal muscle

Figure 9.11 (a) First developed system providing electrical stimulation to myoblasts.⁷³ (b) Schematic representation of the experimental set-up devised by Serena *et al.* for electrophysiological stimulation of collagen scaffolds.⁷⁴ (c) Enhanced differentiation of myoblasts (C2C12) cultured on conductive nanofibers scaffolds.⁷⁵ (d) A custom protocol in which the parameters (amplitude, frequency and pulse duration) of applied electrical field are varied every 3 days to enhance myoblast differentiation.⁷⁶ (e) A custom cylindrical bioreactor capable of providing electromechanical stimulation by inflating of a tubing (covered by aligned nanofibers on top of which cells are seeded) and synchronized electrical stimulation.⁶⁹

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tissues *in vitro* and to properly modulate phenotype expression of arising myotubes. In particular, electrical stimulation aims at mimicking the function of the neuromuscular junction that regulates a complex cascade of events – the excitation-contraction coupling process.⁷¹

Despite the fact that electrical stimulation has been used for several decades to study the electrophysiology of skeletal muscle tissue *in vivo* and *ex vivo*,⁷² *in vitro* electrical stimulation of 3D engineered constructs has become more and more studied just in the last fifteen years with ever-increasing interest among the research community. Electrical stimulation is generally applied to 3D constructs (containing skeletal muscle progenitors) in the form of short pulses (pulse duration is in the order of a few ms) at relatively low frequencies (generally in the range 1–100 Hz), whereas the amplitude of the applied electric field is often normalized to the length of the 3D constructs and in the range of 0.1–5 Volt mm⁻¹. In particular, two ranges of frequencies are generally applied to either induce individual rapid twitch contractions (low frequencies <10 Hz, low force generation) or sustained tetanic contractions (high frequencies >50 Hz, highest force generation).

One of the very first pioneer works published reporting electrical stimulation of 3D engineered constructs dates back to 2000.⁷³ In this work, the authors used SCs and fibroblasts to create “myooids” (*i.e.* 3D structures that resemble skeletal muscle tissue and do not require any additional scaffold material) that were then exposed to cyclic electrical stimulation protocols. Electrical stimulation combined with a custom force transducer allowed to determine maximum isometric tetanic force (Figure 9.11a). However, the obtained values were considerably lower than control muscles from adult animals, indicating that the myooids, despite showing some morphological and functional features similar to native tissue, arrested their development to an early state.

Another work from Serena *et al.* has described the beneficial effect of electrical stimulation over the myogenic potential of SCs.⁷⁴ Muscle progenitors were embedded into collagen hydrogel porous scaffolds and exposed to cyclic electrical pulses with a duration of 3 ms, frequency of ~33 mHz and 70 mVcm⁻¹ amplitude (Figure 9.11b). The results showed that SCs cultured under electrical stimulation expressed more desmin and MyoD, two specific markers involved in muscle differentiation.

Another feature recently studied, which seems to play an important role during muscle cell differentiation, involves the conductive properties of the scaffolds in which cells are embedded or seeded on top. In the work of Jun *et al.*, conductive nanofibers made of poly(L-lactide-co-3-caprolactone) (PLCL) blended with polyaniline (PANi) were used as substrate to grow C2C12 myoblasts.⁷⁵ The authors showed that, although PLCL/PANi fibers had a minimal effect on the proliferation of myoblasts, they promoted the myogenic differentiation of myoblasts in terms of number and length of myotubes, and significantly increased expression level of myogenic genes such as myogenin, troponin T, and MHC (Figure 9.11c). Such conductive

substrates are extremely interesting and may be used in future studies to enhance electrical stimulation or to study electrical signals during muscle cell differentiation.

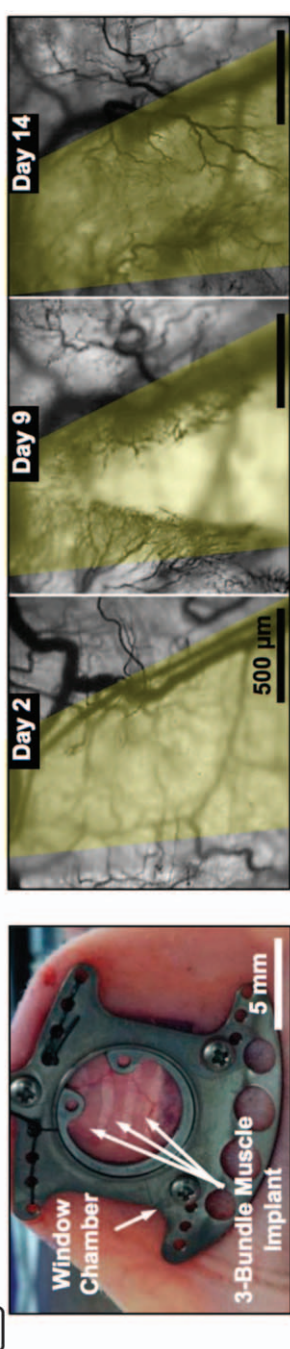
In a recent work by Ito *et al.*, C2C12 cells were magnetically labelled with magnetite cationic liposomes, enabling the authors to concentrate the cells into highly packed structures using magnetic force.⁷⁶ After 3 days of *in vitro* culture, electrical stimulation was activated and continued until day 14. Interestingly, the authors changed the parameter of electrical stimulation every 3 days to optimize C2C12 differentiation and maximize peak twitch force (Figure 9.11d).

9.7 *In Vivo* Studies

In vivo, the process of muscle regeneration after injury is influenced by a multitude of factors involving local inflammatory body responses that induce the expansion and differentiation of resident myogenic precursor cells into new muscular tissue.¹⁸ Unfortunately, this inherent mechanism of self-healing fails in case of massive loss of muscular tissue (Volumetric Muscle Loss, VLM) or in presence of congenital myopathies.

TE approaches for skeletal muscle regeneration can be considered successful when the (bio)implant functionally integrates with the adjacent muscular tissue and neurovascular networks of the host, restoring the mechanical bridging, the transmission of contraction and the force generation in the injured muscle.

In the last decade, many *in vivo* studies focused on the effect that different TE strategies have on muscular regeneration. Among them, cell-centric approaches rely on the exogenous delivery of stem or progenitor cells to enhance the chances of muscular regeneration by increasing the number of cells that take active part to the process and/or by inducing paracrine signaling within the host injured microenvironment that promotes healing processes. SC-derived myoblasts have been considered promising candidates for muscular regeneration cell therapies,⁷⁷ and the characteristics and regenerative potential of different SC sub-populations, maturation states and delivery methods are under continuous investigation.¹² For example, Cerletti *et al.*⁷⁸ isolated a subset SC population, labelled as CSM4B, from adult mouse skeletal muscle, demonstrating how it can increase muscular regeneration rate and function recovery when injected intramuscularly in immunocompetent dystrophin-deficient mdx mice and cardiotoxin-injured wild-type mice. More recently Lorant *et al.*⁷⁹ identified a subset of human adult muscle stem cells (CD46⁺ hMuStem) with high potential for tissue engraftment and muscle regeneration when injected in cryo-damaged mouse muscle. Despite these promising results, the difficulties associated with the isolation of SCs, their *in vitro* maintenance and expansion, as well as their inability to migrate through vasculature and the subsequent necessity for local injection, limit their clinical application.⁸⁰ Besides muscular derived cells, other types of cells, like pericytes,⁸¹ mesoangioblasts⁸² or



mesenchymal stem cells of different origins, such as amniotic fluid,⁸³ synovial membrane,⁸⁴ bone marrow⁸⁵ or adipose tissue,⁸⁶ showed the ability to take part in myogenesis processes when injected locally or systemically.

These findings suggest that cell-based TE strategies are promising therapeutic tools, especially in dystrophic patients.¹ Nevertheless, pure cell-centric approaches may be inefficient in restoring muscular function in VML injuries, independent from the cell type used, due to the large volume of the defect.⁸⁷ In these cases, the use of scaffolding biomaterials becomes essential in guiding the 3D, macroscopic regeneration of lost muscular tissue. For example, Yang *et al.*⁸⁸ showed that aligned muscular cells seeded on nanopatterned poly(lactic-co-glycolic acid) (PLA) scaffolds preserved a higher myogenic potential as opposed to cells seeded on un-patterned scaffolds, demonstrating how properly architected scaffolds can act as guidance for cellular organization also *in vivo* (Figure 9.12a). In another study, Juhas *et al.*⁸⁹ engineered aligned muscle bundles by culturing a neonatal rat myogenic cell population in fibrinogen/Matrigel gels kept under uniaxial tension. Their study revealed how the *ex vivo* maturation of the engineered muscle increases its *in vivo* myogenesis potential not only in terms of functionality (diameter of myofibers, cross-striations and contractile force) but also in terms of neovascularization (Figure 9.12b). Besides these findings, the study also revealed how the absence of a pre-vascular network within the construct determines an initial damage of the engineered tissue after implantation due to hypoxia, confirming the critical role of neovascularization and vascular anastomosis. Indeed, the formation of vessels and capillaries within bio-implants represent one of the major limits to the overall dimension of implantable engineered tissues.⁹⁰ In this respect, the inclusion of endothelial cells in engineered muscular constructs and their *ex vivo* maturation was shown to increase the efficiency of neovascularization and anastomosis *in vivo*.⁵² This approach resulted in efficient implants of millimetric dimension, but its suitability for engineered muscles of larger dimension needs to be proved.

Figure 9.12 (a) Muscle patches made of flat or nanopatterned PLA seeded with muscle derived mouse cells.⁸⁸ (b) Muscle bundles made of myogenic precursor cells and fibrinogen/Matrigel ECM (1.25 cm×3 mm) implanted in a dorsal skinfold chamber in nude mice. Vascularization of the bio-implant could be visible after two weeks.⁸⁹ (c) Biopsies of acellular biological scaffolds implanted in patients with VML injuries after 24–28 weeks. Immunohistochemistry and Immunofluorescence characterizations reveal the formation of desminin+ muscle fibers, the presence of CD146+ perivascular stem cells and β -III Tubulin+ nerve bundles.⁹³

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Alternatively, the use of acellular scaffolds can circumvent the limitations associated with neovascularization issues. In this case, the implant will be populated and invaded *in vivo* by adjacent cells and vessels, avoiding the arising of necrotic areas. Although cell-seeded scaffolds showed a more pronounced muscle regenerative potential with respect to acellular scaffolds,⁹¹ the latter do not suffer from drawbacks such as cell sourcing, cell death after implantation and more severe regulatory restrictions. This muscular TE strategy has been the first to reach clinical application. The most promising acellular scaffolds used for muscular regeneration *in vivo* are of biological origin,¹ obtained by decellularization processes of mammalian tissues.⁹²

Dziki *et al.*⁹³ recently demonstrated that decellularized scaffolds obtained from different porcine tissues, namely intestinal submucosa, urinary bladder and dermal ECM, induce a similar rate of neovascularization and new muscle formation when implanted in VML injuries in human patients (Figure 9.12c). Further, treated patients improved their range-of-motion and muscle force production.

9.8 Conclusion and Future Directions

Although skeletal muscles possess a capacity for self-regeneration in response to small injuries, treatment of volumetric muscle loss still remains a big challenge. Difficulties are even higher when together with muscle tissue, blood vessels and nerves should be reconstructed. In such cases, TE could be a promising alternative to standard medical procedures based on autologous muscle transplantation. Different *in vivo*, *in vitro* or *in situ* TE approaches have been studied to drive regeneration of the skeletal muscles. Independently from the cell type used, pure cell therapy may be insufficient in treatment of critical size defects. Therefore, several natural, synthetic or hybrid bioactive scaffolds alone or enriched with stem cells and/or growth factors were designed and tested for muscle engineering addressing also need for neotissue vascularization and innervation.

In summary, current achievements in muscle TE are very promising, especially in development of *in vitro* models. However, before this approach would be widely applied in the clinic, optimal strategies for successful skeletal muscle regeneration still have to be defined. These strategies should be tailored to the specific patient and specific clinical situation, based on better understanding of the healing and remodeling processes occurring in human body.

In the future, further development of multifunctional, hybrid and bioactive scaffolds inducing endogenous cascade of muscle regeneration by providing natural ECM-like conditions and selectively mobilizing and activating proper host or delivered cell populations is needed. It is crucial that they will not only promote the growth and maturation of muscle cells, but also stimulate vascularization and innervation, which is essential for clinical application, especially in the case of VML. Engineered constructs could be

pre-vascularized *in vivo* though implantation around a vascular pedicle. The needed vascular network might be also formed *in vitro*, for instance, using advanced 3D bioprinting technologies combining different bioinks and cell types. Moreover, 3D bioprinting shows high potential in biofabrication of patient-specific neuromuscular junction essential for restoring contractive activity and creating functional muscle.

It is already proven that mechanical and electrical stimulation can be beneficial for muscle TE. Therefore, future studies should be also focused on developing strategies of applying proper mechanical loads and electrical signals to the cells *in vitro*, using advanced bioreactors and conductive scaffolds, or even *in vivo*, by the development of noninvasive stimulatory methods.

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CHAPTER 10

3D Tissue Modelling of Orthopaedic Tissues

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10.1 Introduction

Bone is a dynamic tissue. In spite of its huge regenerative capacity, bone augmentation is often required in cases like congenital or acquired defects, or in diabetic patients with compromised healing capacity. Reconstruction of damaged or diseased orthopaedic tissues is still a significant global healthcare challenge. Presently-practised bone reconstructive procedures use autogenous bone grafts, allogeneic bone or synthetic materials. Autogenous bone grafts are considered the ‘gold standard’ to date, but the downsides of this include morbidity at the donor site, restricted availability and the need for a second surgery to harvest the bone.¹ In case of allografts, immunogenicity and transmission of diseases are the possible risk factors.² And when it comes to synthetic materials, lack of proper integration, leading to subsequent bone resorption are the major drawbacks.^{3–5} To overcome the above drawbacks, a transition is required from the use of permanent, inert metals to biodegradable tissue engineered composites intended to mimic the composition of bone.

Regardless of the broad exploration in this field, apart from the ‘gold standard’ autograft bone presently no accessible bone substitute materials

can offer osteoinductive and osteogenic assets in one single material. For that reason, the essential idea is to apply the concepts of tissue engineering, *i.e.* to interplay with the cells, scaffolds and biological molecules to form a “tissue engineering construct” (TEC), which can promote bone repair and regeneration.^{6,7}

In our body, cells grow and mature in a 3D environment. Based on this thought, it is evident that a similar 3D microenvironment of the tissue should be replicated even in the *in vitro* models for a better physiological cell response.^{8–11} Fortunately, with the advent of 3D printing technology, also called additive manufacturing (AM), tissue engineering constructs (TECs) with diverse or desired structures can be acquired by altering the computer-aided design (CAD) model using computed tomography (CT) or magnetic resonance imaging (MRI) data.¹² This technique gives us the scope to prototype complex designs and unique profiles which are specific to the patient.^{13,14}

On the other hand, even after developing and validating physiologically relevant 3D *in vitro* systems in the basic research of recent times, the actual strategic application and new expertise on the actual patients seems to be a far dream. This chapter concentrates on the concepts of 3D bone tissue modelling and also offers present-day strategies and future standpoints to bring this into clinical practice.

10.2 Tissue Engineering Strategies for Orthopaedic Tissues

Tissue engineering of bone combines the values and ethics of engineering and life sciences to overcome shortcomings of old-style osseous regeneration techniques used in orthopaedics.¹⁵ Three arms of the 3D tissue modelling are the cells, scaffolds and biological cues and these are often considered separately as a result of the technical and regulatory translational challenges of combining materials, cells and biologics into one therapeutic.¹⁶ Table 10.1 summarizes the cells, scaffolds and biological cues which are mandatory to build up a regenerative biomaterial scaffold in order to achieve a successful bone tissue constructs.^{17–20}

There are two main approaches: the first is the cell-based approach in which the cells alone or in combination with scaffolds are used; the second is scaffold-based where a scaffold alone or the scaffold seeded with cells are implanted. Though the two different strategies are mentioned, it is difficult to draw a clear margin between them.

10.2.1 Cell-based Approach

Cells are the key role players involved in tissue development and homeostasis; this is the reason they are most commonly utilized in tissue engineering. The key regulators in bone deposition, modelling, and remodelling are the osteoblast and osteocyte cells. Hence, for a successful cell-based bone

Table 10.1 Use of cells, scaffolds and biological cues in tissue engineering.^{17–20}

Cells	• Adipose-derived stem cells (ASCs) • Adipose-derived stromal vascular fraction (SVF) • Bone marrow-derived mesenchymal stem cells (BM-MSCs) • Dental Pulp Derived Stem Cells • Embryonic stem cells (ESCs) • Induced pluripotent stem cells (iPSCs) • Umbilical cord blood mesenchymal stem cells (CB-MSCs)
Scaffold	Materials used: • Polymer: natural – chitin, collagen, chitosan, chondroitin sulphate; synthetic – poly (lactic acid) (PLA), poly (ϵ-caprolactone) (PCL), poly (glycolic acid) (PGA), poly (lactic-<i>co</i>-glycolic acid) (PLGA) • Ceramics • Composites Fabrication methods: • Conventional • Advanced
Biological cues	• Biochemical stimuli – Bone morphogenic proteins (BMPs), Transforming Growth Factor-β proteins (TGF-βs), Insulin-like Growth Factors: IGF-I and IGF-II, Calcium phosphate, hydroxyapatite coatings. • Biophysical stimuli – bioreactors, porosity, nanotopography, surface roughness <i>etc.</i>

regeneration, osteoblasts and/or their precursors are taken into consideration.^{21–23} There are three major cell therapeutic strategies: (1) implantation of isolated cells, (2) implantation of a construct fabricated from cells and scaffolds, or (3) *in situ* bone regeneration *via* native cells.¹⁸

10.2.2 Scaffold-based Approach

Scaffolds are 3D biocompatible structures which should have the ability to mimic extra cellular matrix (ECM) properties such as cell differentiation, mechanical strength, and protein adsorption *via* biological signalling, and provide a platform for cell attachment and stimulate osseous tissue formation. There are many biomaterials available (as shown in Table 10.1) but choosing the right material is very important—this choice depends on the material property, its biological interactions, structural features and the end result to be achieved.^{24,25} Pre-requirements for a scaffold are mentioned in Table 10.2.²⁶

Materials used as scaffolds comprise polymers (natural and synthetic), ceramics and their composites. Following the *in vivo* implantation of the scaffold, the cells migrate into it and adsorption of the tissue fluid protein takes place. The architecture and porosity of the scaffold should be designed in such a way that it delivers easy implantation, similar mechanical strength to bone, cell attachment and differentiation along with the exchange of

Table 10.2 Ideal requirements of a scaffold for tissue engineering.²⁶

-
1. 3D fully interconnected porous architecture for cell differentiation, nutrient exchange and removal of metabolic waste
 2. Optimized surface characteristics for the cell attachment, proliferation, maturation and differentiation
 3. Biocompatibility and biodegradability with a controllable degradation kinetics
 4. Mechanical properties matching with the tissue of interest
 5. Stress-free and efficient reproduction of the scaffold structure
-

nutrients and metabolic wastes. Additionally, the design should permit vascularization and new bone ingrowth corresponding to the biomaterial degradation without losing its properties once sterilized.⁵

Conventional approaches for scaffold fabrication include freeze drying, fiber spinning, gas forming, particulate leaching, solvent casting, melt moulding, phase separation *etc.*^{27,28} A lot of drawbacks exist for these fabrication approaches; these include a lack of clear-cut control over the pore diameter and their spatial distribution along with fully interconnected architecture of the scaffolds.^{29–31} Moreover, most of these methods utilize organic solvents and their residues pose a potential toxicity to the cells thus biocompatibility of the scaffold is significantly reduced.³²

10.2.3 Additive Manufacturing (3D Printing)

With the advent of 3D printing or Rapid Prototyping (RP) into the field of tissue engineering, the loopholes of the conventional fabrication procedures are taken care of. The concept behind the RP process is to design a 3D digital model which is utilized for scaffold generation. This digital model can be either drawn using CAD or can be generated using the data generated by 3D imaging, *i.e.* CT or MRI images. Then the 3D digital model should be converted to a stereolithographic (STL) file which represents the 3D structure in the form of multiple horizontal planes. Next the RP machine utilizes this STL file to fabricate a 3D scaffold in a layer by layer manner to form a solid object. There are various AM techniques which include stereolithography (SLA), Selective Laser Sintering (SLS), and Fused Deposition Modelling (FDM) (Figure 10.1).^{28,33–35} The merits of 3D Printing for scaffolds fabrication are mentioned in Table 10.3.³⁶

10.2.3.1 3D Bioprinting

Apart from printed acellular scaffolds, AM approaches are also used to discover opportunities in fabricating scaffolds with live cells and tissues—this is termed bioprinting.²⁵ Bioprinting can be defined as a “computer-aided allocation of living and non-living materials in a layer by layer organization to form a bio-engineered structure for the purpose of regenerative medicine and biological studies.”³⁷ Bioprinting promises fast, on demand, and

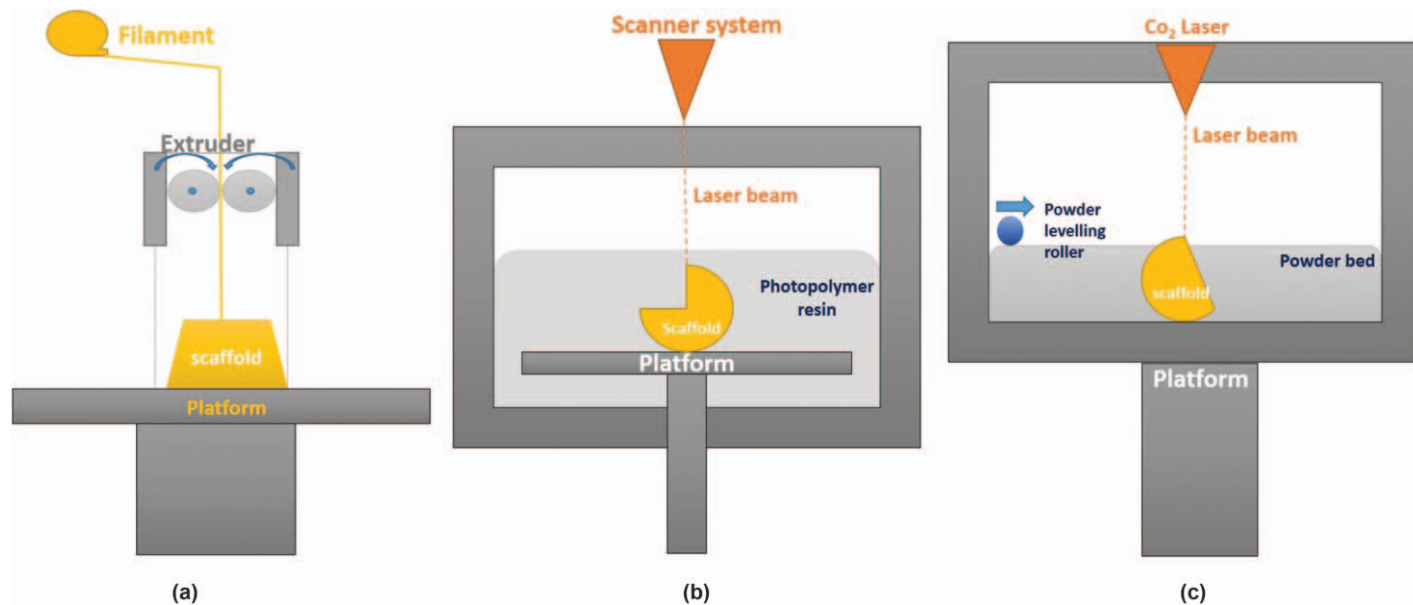


Figure 10.1 Scheme of (a) FDM, (b) stereolithography (SLA), and (c) selective laser sintering (SLS) 3D printing techniques

Table 10.3 Merits of scaffold fabrication *via* 3D printing technique.³⁶

Properties	Advantages
Variability	Higher variability in design aspect with improved biocompatibility and targeted degradability
Formability	Possibility to form into various complex shapes and volumes
Practicability	Features can be tailored for patient-specific applications
Controllability	Control on chemical and physically properties
Applicability	Possibility of designing the precise matrix configuration for adequate nutrient and waste exchange
Flexibility	Flexibility to produce an exact replica as well as to scale up. Flexibility to optimize the parameters to provide a surface area for cell attachment
Design	Design a composite scaffold should be done in such a way that the ceramic components neutralize the degradation by-products of the polymer.
Mass delivery	The capacity to incorporate uninterrupted delivery of necessary hormones and nutrients
Surface properties	The ability to alter the parameters like pore diameter, distribution and overall porosity to have a command on the final mechanical properties.

automated manufacturing of high-resolution constructs. The process involves the use of 3D printing technologies to deposit cells or biological factors into predefined shapes and sizes.³⁸ Bioprinting permits stringent control on placement of cells within matrices and enables the arrangement of biological materials within composite, hierarchical structures and patterns. This promises new opportunities to fabricate reproducible, patient-specific grafts with low risk of immune rejection.

10.3 3D Modelling

10.3.1 Analysis of Patient Defect

Main concern when developing an efficient bone graft substitute the noteworthy variation in each patient's bone anatomy. Additionally, the size and shape will be based on the defect type. To obtain a better outcome, the defect characteristics should be taken into consideration while graft designing and fabrication.^{39,40} To obtain these details accurately, the crucial step is to acquire an image of the defect. The two most widely used imaging modalities are the CT and MRI scans.⁴¹ This is the most fundamental step because inaccurate recording of the defect can lead to malunion, unstable biomechanics, variations in stress distribution, failure of the surgery, need for a revision surgery, *etc.*^{42,43} Kurtz *et al.*, in a survey on total hip replacement surgeries in USA, reported that there are more than 40 000 revision surgeries which are carried out every year.⁴⁴ To avoid the above failures most studies have reported using high-resolution CT images and increasing the number of slices.^{45,46}

10.3.2 Virtual Reconstruction of Defect

The images which are acquired by the imaging modalities cannot be used straight away as they are 2D slices and necessitate processing before uploading to the 3D printer. The process of segmentation is the first step in image processing, in which the pool of blood is removed from the tissue anatomy. This step requires various software such as Mimics, Osiri X, Blender, 3D slicer, Meshmixer *etc.*, as well as a lot of manual work including drawing, erasing, regional thresholding, *etc.* to interpolate the data between the slices.⁴⁷ The next step is modelling. In this step the CT and MRI data that are 2D images—*i.e.* DICOM (Digital Imaging and Communication in Medicine) files—are then converted into a 3D virtual model—*i.e.* STL (Stereolithography or Standard Tessellation Language)—file format. This file conversion requires a number of steps, namely segmentation of the image, 3D mesh generation and reconstruction to finally obtain the 3D model.⁴⁸ The 3D printer receives the virtual models and slices them into digital cross-sections to use as a guideline during printing.⁴

10.3.3 FEM Analysis for High Efficiency and Accuracy

Development of a tailored intervention in the field of orthopaedics is mandatory to meet present-day needs.^{49–51} So, it is of great importance to accurately plan and implement the procedure to meet patient-specific needs. Regrettably, it is difficult to preoperatively predict the postoperative mechanical aspects of the graft and it is unethical to utilize the surgical procedures as a measure of stress.^{52,53} Various methods like finite element analysis and personalized surgery^{54,55} are developed to assess and predict the bone morphology and load transfer prior to the treatment to have an accurate preoperative design.^{56,57} For a perfect and successful graft fixation a close representation of the patients defect geometry as well as mechanical factors should be taken into account during the preoperative planning. The patient-specific geometrical model will provide the perceptions of the stress distribution in bones.^{58–60}

10.3.4 Prototype Fabrication

Prototype fabrication is the procedure of making a 3D object from a virtual 3D model. Then the printer utilizes this 3D virtual data to generate a physical 3D model, which is a 1:1 replica of the computer model. In this step, the physical model is generated by transferring the STL file to the printer. Based on the material and property requirement, the type of 3D printing process is opted.⁴¹ After the design format STL file is read by the 3D printer, it creates a series of cross-sections to build the model in a layer by layer fashion with powder, liquid, *etc.* A few 3D printers use multiple materials to print the constructs while others utilize those materials as supports while printing the

model. Once the printing is done, the supports can be removed or dissolved to obtain the final object.⁶¹ Depending on the type of RP process selected, to obtain the final 3D prototype some post-processing is required. This is done to enhance the surface aesthetics and characteristics by removal of the powder sediments and support structures with various techniques such as bead blasting, tumble finishing, painting, *etc.*⁴¹

A few techniques of 3D printing, such as SLS, SLA and FDM, focus on designing and generating scaffolds, but would not allow cell printing.⁶² After the sterilization procedures, proper loading of the cell type onto a scaffold either in static conditions by intubation or in dynamic conditions by using a bioreactor for tissue growth *in vitro* is required. On the other hand, a sterilized 3D printed scaffold without cells could be implanted into human body with an intention to recruit and reorganise host cells in the body.⁶³

To explore the possibility of printing cells along with the scaffold, the 3D bioprinting technique was developed. The soul of this 3D bioprinting technology is the bioink, which consists of cells and biological cues for cell function and tissue formation.⁶⁴ The components of extracellular matrix (ECM) like collagen, elastin, fibrin *etc.* are used as bioinks.^{65,66} Presently the materials used as bioink are principally built on the basis of natural and synthetic polymers.^{67–70} As the final 3D model is printed along with cells it can be intubated for *in vitro* tissue growth or can be directly implanted.⁷¹

10.4 Case Reports

10.4.1 Case Report 1

Well-characterised and clinically relevant animal models are essential to create proof-of-principle pre-clinical data necessary to move from novel therapeutics to clinical application. To study various treatment possibilities on large volume segmental bone loss, a research group at the Queensland University of Technology, Australia came up with a defect model.^{72,73} To study the association between the cells and growth factors (bone morphogenic proteins) on a medical-grade poly(caprolactone-tricalcium phosphate) (mPCL-TCP) scaffold, they established a 3 cm critical sized defect model in sheep tibia.^{74,75} With the FDM technology, they fabricated the scaffold. They utilized the CAD design for maintaining the structural parameters like 350–500 μm pore size, 100% pore interconnectivity and a 0/90° lay-down pattern.³⁶ After the removal of the periosteum, a 6 cm tibial defect is made was the diaphysis. Later, the mPCL-TCP scaffold with the same dimensions is loaded with platelet-rich plasma (PRP) and recombinant human bone morphogenetic protein-2 (rhBMP-2) to bridge the defect and the plate fixation is done. Radiographic analysis at 3 months postoperative illustrated newly formed radio-opaque mineralised tissue bridging of the defect site, thus revealing the potential for bone regeneration even in such large defects.^{36,76}

10.4.2 Case Report 2

Evaluation of the osteogenic potential of the porous titanium implants, fabricated *via* selective electron beam melting (SEBM) was done by implanting into defects in the frontal skull of 15 domestic pigs. Postoperative radiographic and histomorphometric analyses at the bone implant interface are done to evaluate osseous tissue ingrowth on day 14, 30 and 60. During the study period the bone ingrowth was remarkable: on day 14 it was about 14%, which increased to 30% and 46% on day 30 and 60 respectively. Thus this work proves that the porous scaffolds are the good candidates for orthopaedic applications.⁷⁷

10.4.3 Case Report 3

Three porous titanium implants are generated by the selective laser melting (SLM) technique for evaluation of the effect of various pore sizes (309, 632, and 956 μm , designated as P300, P600, and P900 implants, respectively) and with constant porosity of 65% on *in vivo* bone ingrowth in rabbits. The basic structure was a diamond lattice. Microfocus X-ray CT was used to assess the porosity. Bone ingrowth was measured at 2, 4, and 8 weeks. At 2 weeks, the P600 implant revealed significantly higher bone ingrowth compared to others and the P300 implant revealed lower bone ingrowth than the other implants at 4 weeks. This concludes that an average pore size of 632 μm encourages rapid bone ingrowth, adequate fixation and mechanical strength, and is thus suitable for orthopaedics.⁷⁸

10.4.4 Case Report 4

Evaluation of the efficiency of various citric-acid-based polymers—(CABP)/hydroxyapatite (HA) composites, poly(1, 8-octanediol citrate)-click-HA (POC-Click-HA) and cross-linked urethane-doped polyester-HA (CUPE-HA)—in comparison with autologous bone grafts to repair skeletal bone defects was done. CABP-HA disc-shaped scaffolds (65 wt% HA with 70% porosity) were used as bare implants without the growth factors or cells to repair 4 mm diameter calvarial defects in the rat. Defects were either left empty (negative control group), or treated with CUPE-HA scaffolds, POC-Click-HA scaffolds, or autologous bone grafts (AB group). Radiographic and histological data exhibited a noteworthy improvement in osteogenesis of the defects treated with CUPE-HA scaffolds in comparison to POC-Click-HA scaffolds. Both, POC-Click-HA and CUPE-HA scaffolds presented superior trabecular thickness and bone density in comparison to the control group after 1, 3, and 6 months of implantation (Figure 10.2). These results justify the capability of CABP-HA implants as osteogenic and off-the-shelf existing options in orthopaedic remodelling.⁷⁹

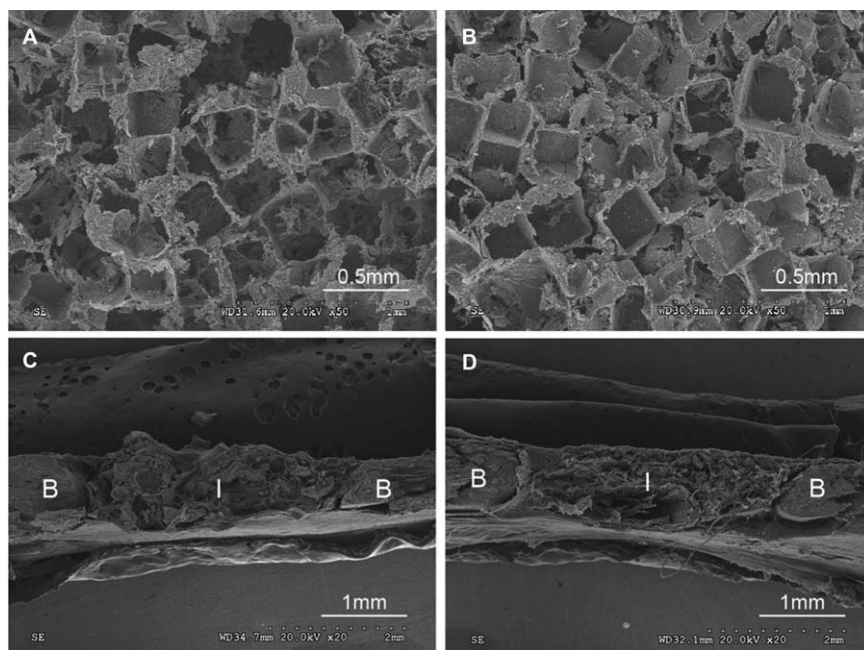


Figure 10.2 Left Panel: Representative scanning electron microscope (SEM) images of bare cross-linked urethane-doped polyester – hydroxyapatite (CUPE-HA) scaffolds (A) and (B) poly (octanediol citrate) – click – hydroxyapatite (POC-Click-HA) scaffolds (60 wt.-HA and 70% porosity); and (C) CUPE explants and (D) POC-click-HA explants 6 months after implantation in a 4 mm rat calvarial defect (magnification 503) (I: implant; B: bone). Right Panel: Microcomputer tomography (micro CT) reconstructed 3D images of 4 mm rat calvarial defects treated with (a–c) negative control (untreated defects) (CON), (d–f) autologous bone (AB), (g–i) cross-linked urethane-doped polyester-hydroxyapatite scaffolds (CUPE-HA), and (j–l) poly (octanediol citrate)-click-hydroxyapatite scaffolds (POC-Click-HA) 1, 3, and 6 months' post-implantation. Reproduced from ref. 79 with permission from Springer Nature, Copyright 2014.

10.4.5 Case Report 5

Evaluation of the osteogenic potential of the albumin scaffold was done. Polymerization of the human, bovine, and porcine albumin was done by microbial transglutaminase and later freeze-dried to form albumin tissue scaffolds. Porous structure and mechanical properties of the albumin scaffold were evaluated by scanning electron microscope and mechanical testing analysis respectively. Later the human MSCs (hMSCs) were seeded and cultured in the albumin scaffold, which revealed excellent osteogenic differentiation of the stem cells. These results comment on the favourability of albumin tissue as a biocompatible scaffold with adequate mechanical properties.⁸⁰

10.4.6 Case Report 6

Bioprinted bone marrow-derived hMSCs in a poly(ethylene glycol) dimethacrylate (PEG-DMA) scaffold was evaluated for stimulating osteogenesis. hMSCs suspended in PEGDMA were printed along with nanoparticles of bioactive glass (BG) and hydroxyapatite (HA) under simultaneous polymerization to obtain accurate substrates. After 21 days of culture, cell viability ($86.62 \pm 6.02\%$) and compressive modulus (358.91 ± 48.05 kPa) were more in the case of hydroxyapatite HA when compared to bioactive ceramics. Alkaline phosphatase activity and collagen production were correlated with the gene expression which inclined more towards hydroxyapatite composites. Thus, to conclude, bioprinting of HA composites with hMSCs is more efficient compared to BG in osteogenesis.^{38,81}

10.5 Concept to Clinic

10.5.1 Transforming Strategies of Bone Tissue Engineering from Lab to Patient

Orthopaedic problems are extremely widespread and are the main reason behind patient's pain, illness and disability. These problems fall under the second-most common reason for consulting a general practitioner, accounting for about 25% of total illness cost and 15% of primary care. There is a huge impact of bone trauma due to the failure to restore an injured limb post-surgically. This is statistically shown in that only 28% of patients return to their work after being treated for severe tibial open fractures.⁸² In addition, tumour resection is also another major causes of large bone defects. A huge number of procedures are introduced for generating bone implant material, and this will continue further in the future. The existing bone grafting market already is about 2.5 billion USD each year and is estimated to surge by 7–8% per year.⁸³

Hopes and expectations were extremely high, especially after tissue engineering came into the picture as it is able to substitute natural organs with similar replacement organs. There were 3300 scientists and support staff with more than 70 companies with an expenditure of over 600 million USD were involved in tissue engineering research in the beginning of 2001.⁸⁴ There was a huge increase in the funds from 2.36 billion USD to 614 billion USD during 2003 to 2006 by the US National Institutes of Health (NIH).⁸⁵ Publications on tissue engineering also increased from 400 to 900% from the year 2000 to 2008. It has been 30 years now, but the translation of the bone tissue engineering technologies in to routine clinical practice has still not taken place. This could be because of the complexities in these strategies making it difficult to reach the clinical level. This gap is called a “Valley of Death” as a huge number of ventures “die” due to lack of funds as shown in Figure 10.3.⁸⁶

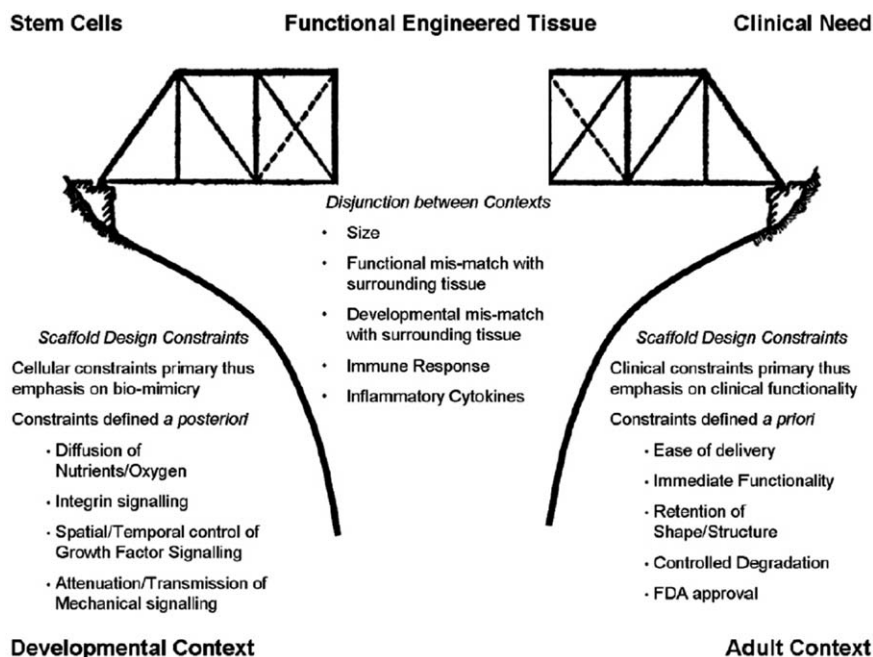


Figure 10.3 Bone tissue engineering strategies rely on three-dimensional scaffolds that constitute an inductive/conductive extracellular microenvironment for stem cell function as well as a delivery vehicle and 3D scaffold of clinically relevant properties and proportions. In fulfilling these dual criteria, the biomimetic scaffold plays a critical role bridging the gap between the developmental context of stem cell mediated tissue formation and the adult context of injury and disease. Reproduced from ref. 86 with permission from Elsevier, Copyright 2008.

10.5.2 Bridging the Breach Between the Research and Clinical Applications of Tissue Engineering

To bring up the concepts of tissue engineering into the daily practise, the scaffold design should meet the clinically relevant scale and prerequisites. As shown in Figure 10.3., the 3D scaffold should mimic the extracellular microenvironment for cell differentiation. The dual standards of biomimetic scaffold play an important role in bringing the concepts of stem cell driven tissue formation and adult repair of injury and disease together.⁸⁶

The key role players in bringing the research and clinical practice together are the design and manufacturing technologies. Coming up with a decent manufacturing process and practical designs can bridge the valley of death. There is an immense need to understand the present-day clinical needs to achieve applications of research on the patients. To bridge the valley of death, the scaffold should meet the following requirements: FDA approval, cost-effective manufacturing, easy handling, sterilizable, distinguishable from newly formed bone *via* radiographs, with minimal surgical procedures.^{36,87,88}

10.6 Future Perspectives

To boost the formation of bone and increase the efficiency and safety, cells, scaffolds and gene therapy are utilized in regenerative orthopaedics.⁸⁹ In spite of the definite data available on the cell sources and their expansion, various issues still need to be optimized, like matrix signalling, biological cues, mechanical properties, *etc.* The focus should be on cell survival, their expansion, required differentiation, vascularization, integration with the native bone *in vivo*. Present-day thoughts on regenerative medicine underlines the assert of biomimetic materials for enhancing and stimulating the native tissue regenerative capacity.⁵

So what is upcoming? With the increase in tissue complexity, various challenges should be taken into consideration like the vascularity and innervation.⁷¹ Along with these, another important aspect for bone reconstruction is the adequate mechanical strength to accept the physiological stresses. To date, there is still a lag phase when it comes to understanding the exact molecular level mechanisms for bone regeneration.

Furthermore, technological advancements should make 3D printing more affordable. A lot of investigations are in need to study the safety issues of the materials used for printing before implanting into the patient. Lastly, further tests should be performed to allow the standardization of optimal parameters for the technique as well as the material and conditions for maturation of tissues in the fabricated construct.⁹⁰

10.7 Conclusion

Advancements in the tissue engineering has once and for all rewritten the background of contemporary medicine. Increasing demand for tissue replacement can be addressed by implementing safety and functionality in cell and tissue engineering. Clearly, there is a prerequisite for innovative, relevant and cost-effective research strategies to bring up tissue engineering in to clinical practice. A balance between biomaterial simplicity and complexity must also be considered, because increasing scaffold complexity does not always lead to increased efficacy in rebuilding tissues *in vivo* and, most importantly, in the clinic. Immense amount of standardization, stringent protocols and pure hard work on extensive research is demanded in this field as it is purely related to future health, happiness, and quality of human lives.

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CHAPTER 11

3D Tissue Modeling of Skin Tissue

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11.1 Introduction

The skin is the largest and heaviest organ, accounting for approximately 16% of human body weight. It is a multilayered soft tissue that covers the whole surface of the body and consists of an epidermis, dermis, and hypodermis (Figure 11.1).¹ The outermost layer, the epidermis, prevents pathogens from entering the body and maintains a moist protective barrier on the surface of the body. Most of the cells in the epidermis are keratinocytes; there are also melanocytes, Merkel cells, and Langerhans cells. The dermis, which is underneath the epidermis, binds tightly to the basement membrane and is composed of fibroblasts and mast cells. Due to the tensile strength and elasticity of the extracellular matrix (ECM), which contains a range of fibers, the dermis cushions the body from external stress and strain. The innermost layer is called the hypodermis or subcutaneous layer and is composed of adipocytes, lipocytes, macrophages, and collagen. The function of the hypodermis is to provide padding and insulation. Skin is a complex, highly organized tissue composed of these constituents, all of which are vital for survival.

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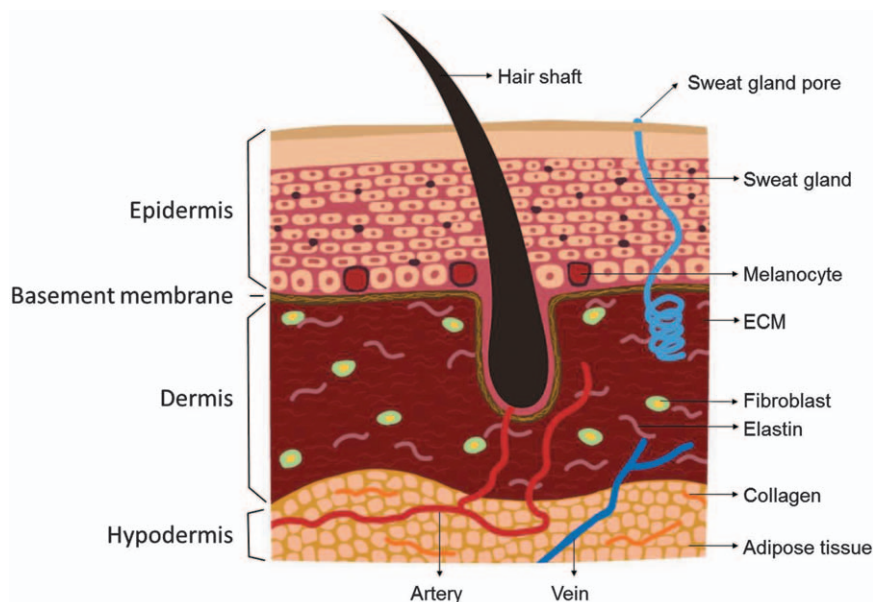


Figure 11.1 Anatomical structure of human skin.

11.1.1 The Need for Skin Substitutes

Skin substitutes can be used to treat skin problems such as burns, cancer, psoriasis, and eczema. Skin substitutes are particularly essential for treating injuries to the full thickness of the skin, or third-degree or fourth-degree burns. However, burn wound treatments are fairly expensive and there are always long waiting lists. In the United States in 2010, the estimated cost of burn treatments was about \$1.5 billion.² In 2016, there were approximately 500 000 burn patients.³ Therefore, we need to develop cost-effective substitutes for artificial skin to meet the increasing demand. In addition to treating skin wounds, skin substitutes can be used to develop new cosmetic treatments and to facilitate drug development. In general, animal testing has been used to study new products; however, growing concern about ethical problems has led to a recent decline in animal testing. For example, animal testing was prohibited for cosmetics by Israel in 2007, followed by the 28 countries of the European Union in 2009.⁴ Hence, we need skin substitutes for the development of efficient and cost-effective skin treatments and to replace animal testing.

11.1.2 Conventional Skin Wound Treatments

Until now, various methods have been used to treat skin wounds. In the case of superficial wounds, the wound is usually dressed with a sterilized gauze or pad. This protects the wound and allows it to heal. Dressing is a cost-effective and convenient method for treating these types of wounds, but is

only suitable for the treatment of thin wounds.⁵ Dressing is not a suitable treatment for deeper wounds, which are usually treated using autografts and allografts. Autografts use the patient's own skin to treat the skin wound, whereas allografts use skin from another individual of the same species. These methods are more effective than dressing in the case of deep wounds. However, there are serious issues with these methods, as there is a limited amount of transplantable skin, and it can be rejected by the patient's immune system.⁶

Skin tissue engineering, which is the study of artificial skin regeneration, was introduced to overcome the difficulties. The representative methods for producing engineered tissue are skin electrospinning and cell sheets. Electrospinning is a method for producing nano-scale meshes from biomaterials. The advantage of this method is that it produces nano-patterned electrospun meshes that have similar architectures to the ECM fibers in the skin dermis.⁷ However, we cannot make thick scaffolds or arbitrarily large pores using this method. The pore size affects cell penetration into the scaffold and angiogenesis. Cell sheets are structures composed of cell and ECM materials, but no biomaterials. These sheets are produced in temperature-responsive culture dishes. Cell sheets that are similar to skin tissue can be produced by the deposition and co-culturing of multiple types of skin cells to form an organized three-dimensional (3D) structure.⁸ However, cell sheet engineering has severe limitations as the resulting sheets are limited in size and do not contain the vascular networks required for artificial skin to be functional.⁹

Recently, many researchers have studied 3D bioprinting technology in the context of skin tissue engineering. In 3D bioprinting, computer templates are used to specify the complex cellular structures with multiple types of cells and biomaterials, and these templates are then 'printed'.⁶ This technology can be used to fabricate cellular complexes that are similar to real skin. There are a number of advantages to 3D skin bioprinting over traditional skin wound treatments. Firstly, as 3D skin bioprinting is an automatic process, it can produce and reproduce skin substitutes accurately and consistently.¹⁰ Secondly, we can use computer-aided design and manufacturing (CAD/CAM) systems to automate the generation of patient-specific skin substitute templates based on each patient's anatomy and physiology.¹¹ There have been numerous studies into the application of bioprinting technologies to the production of functional artificial skin substitutes. In this chapter, we review current bioprinting systems and introduce substitutes to produce functional skin complexes.

11.2 3D Bioprinting System for Skin Tissue Engineering

Three methods are commonly used for skin bioprinting: laser-, inkjet-, and extrusion-based bioprinting (Figure 11.2).¹² Laser-based bioprinting uses

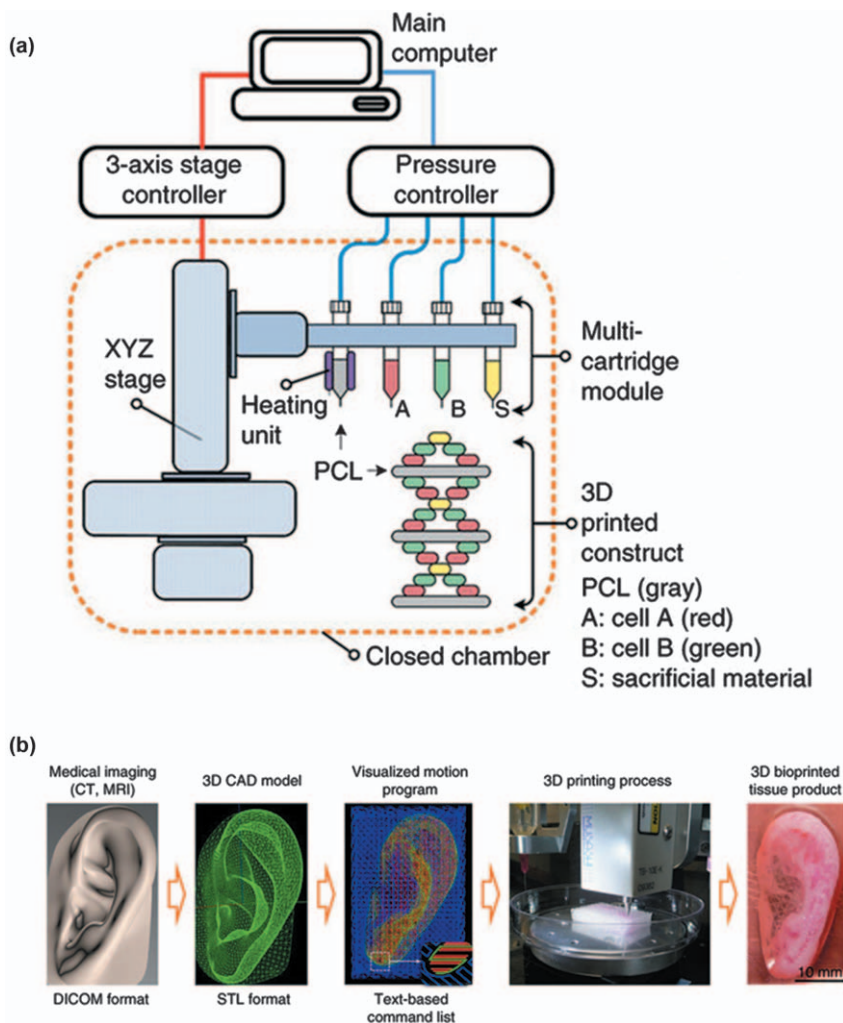


Figure 11.2 Schematic diagrams of laser-, inkjet-, and extrusion-based bioprinting systems.

light energy to deposit droplets containing cells. This method has two main advantages: the ability to position very small volumes of droplets with high resolution and lower limitations on the range of available viscosities in comparison to inkjet-based bioprinting.¹³ Inkjet-based bioprinting uses heating or piezoelectric modules to induce jets of droplets of bio-ink onto the desired target. The inkjet method is fast and cost-effective.¹⁴ Extrusion-based bioprinting uses pneumatic pressure or pistons to print bio-ink. As a result, the bio-ink comes out as a continuous stream. This method can deliver very high densities of cells to a structure.¹⁵

11.2.1 Bio-ink for Skin Printing

Bio-ink is an essential component of the 3D bioprinting process. It is usually composed of living cells and biomaterials. It should have both suitable physical properties for printing and good cytocompatibility to enhance cell adhesion, proliferation, and differentiation. Various types of cells and biomaterials have been used to create advanced full-thickness skin grafts. Table 11.1 summarizes the cell types and biomaterials with special features used by researchers working on 3D bioprinting-based skin tissue engineering.

11.2.2 Cell Source

Skin is basically composed of two layers: the epidermis and the dermis. The epidermis is the outermost layer of skin tissue, and keratinocytes constitute about 90–95% of the cell population in this layer. Fibroblasts are the main cell type in the dermis. These form an ECM structure composed of collagen and elastin.¹⁶ Various kinds of cells have been used in bioprinting-based skin tissue engineering, as shown in Table 11.1. Keratinocytes and fibroblasts are frequently used as cell sources in the production of bi-layered artificial skins. For example, Lee *et al.* used fibroblasts and keratinocytes to print distinctive basic dermal and epidermal-like layers.¹⁷ Advanced artificial skins containing vessels, skin appendages, and sensory receptors have been produced using stem cells such as induced pluripotent stem cells (iPSCs), mesenchymal stem cells (MSCs), amniotic fluid-derived stem cells (AFSCs).¹⁸ Skardal *et al.* conducted an animal study with nude mice that showed that the use of AFSCs results in enhanced neovascularization.⁶ Aasen *et al.* showed that induced pluripotent stem cells can be differentiated into skin-like cells and constructed a multi-differentiated epidermis with hair follicles and sebaceous glands.¹⁹ Progenitor cells have also been used as a cell source. Huang *et al.* showed that epithelial progenitor cells can be used to regenerate sweat glands.²⁰

11.2.3 Biomaterials

Biomaterials are an essential component of bio-ink. They affect both the printability and the skin tissue regeneration. The essential characteristics of biomaterials are: good biocompatibility, nontoxicity, appropriate biodegradability, and no immune response through antigenicity *in vivo*. The mechanical properties of the material must be suitable for the particular transplantation site.²¹ Skin substitutes are usually made from hydrogel materials such as collagen, fibrin gel, and fibronectin, as shown in Table 11.1.²² The materials facilitate continuous secretion of cytokines and growth factors that can improve skin wound healing.²³ In addition, these can mimic the complexity of the ECM structure of skin tissue.²⁴ However, the hydrogels are usually weak in comparison to native tissue. To enhance their

Table 11.1 Various approaches to 3D skin bioprinting, including materials and methods.

Type	Polymer	Cell source	Special feature	Ref
Natural	Collagen	Mouse NIH-3T3 fibroblast, HaCaT keratinocyte	First attempt to generate 3D multicellular constructs resembling simple skin tissue as their native archetype using laser-assisted bioprinting	60
		HFF-1 ^a fibroblast, HaCaT keratinocyte	Air–liquid interface (ALI)-culture and specific crosslinking reagent (NaHCO ₃) could decrease tissue deformation	26
		HDF ^b , HPEK ^c	Newly-developed holistic approach included a cell-compatible ready-to-use bio-ink and used human primary cells	61
		NIH3T3 fibroblast, HaCaT keratinocyte	Evaluation of the subsequent tissue formation <i>in vivo</i> by dorsal skin fold chambers. Cells were arranged in three-dimensional (3D) skin constructs using laser-assisted bioprinting	62
	Hyaluronic acid	HFB ^d , HKC ^e	Extruded micro-strands of collagen were frozen immediately upon contact with a cryogenic plate, to easily control the pore size and reduce the range of shrinkage	34
		AFSC ^f	<i>In situ</i> fast photo crosslinkable heparin-conjugated hyaluronic acid hydrogel improved the sustained release of growth factor	37
	Alginate	NIH3T3 fibroblast, HaCaT keratinocyte, hMSC ^g s	Alginate hydrogel enabled printing of well-defined cell structures	29

Natural composite	Gelatin/Chitosan	HFF-1 fibroblast	Polyelectrolyte gelatin-chitosan hydrogel was extruded to improve biocompatibility, vascularization, and antimicrobial activity	28
	Collagen/Alginate	NIH3T3 fibroblast, HaCaT keratinocyte	Outer collagen and inner alginate caused good structural stability (core shell structure) with co-extrusion of collagen/alginate and cryogenic layer-by-layer processes	21
	Gelatin/Silk fibroin	CFF ^h	Gelatin-sulfonated silk composite scaffold containing fibroblast growth factor-2 (FGF-2) improved epidermis regeneration and dermal vascularization	38
	Gelatin/Alginate/Fibrinogen	Mouse NIH 3T3 fibroblast, HDFa	Special bio-ink formulation with a mixture of natural composites, used to achieve proper gel rheology to make a scaffold-free epithelium	63
Composite	PCL/Collagen	HDF, HEK	Extrusion dispensing module created a collagen-based construct with a PCL mesh and an inkjet based dispensing module was used to construct uniformly distributed keratinocytes. Functional transwell system provided porous 3D construct comprising PCL	35

^aHFF-1: Human foreskin fibroblast-1.
^bHDF: Human primary dermal fibroblast.
^cHPEK: Human primary epidermal keratinocyte.
^dHFB: Human fibroblast.
^eHKC: Human keratinocyte.
^fAFSC: Amniotic fluid-derived stem cell.
^ghMSC: Human mesenchymal stem cell.
^hCFF: Child foreskin fibroblast.

mechanical properties, composite materials composed of hydrogels and polymers such as poly-L-lactic acid (PLLA), chitosan, and poly-caprolactone (PCL) have been introduced.²¹

11.2.4 Basic 3D Skin Bioprinting Technique

3D bioprinting can produce artificial skin tissue with biomimetic multi-layered architectures by laminating skin cells including fibroblasts and keratinocytes, which are the main components of the dermis and epidermis (Figure 11.3). To deliver living cells, a hydrogel, which can also induce the release of growth factors and cytokines, is usually applied as bio-ink.²³ Collagen hydrogel has frequently been used in skin tissue engineering

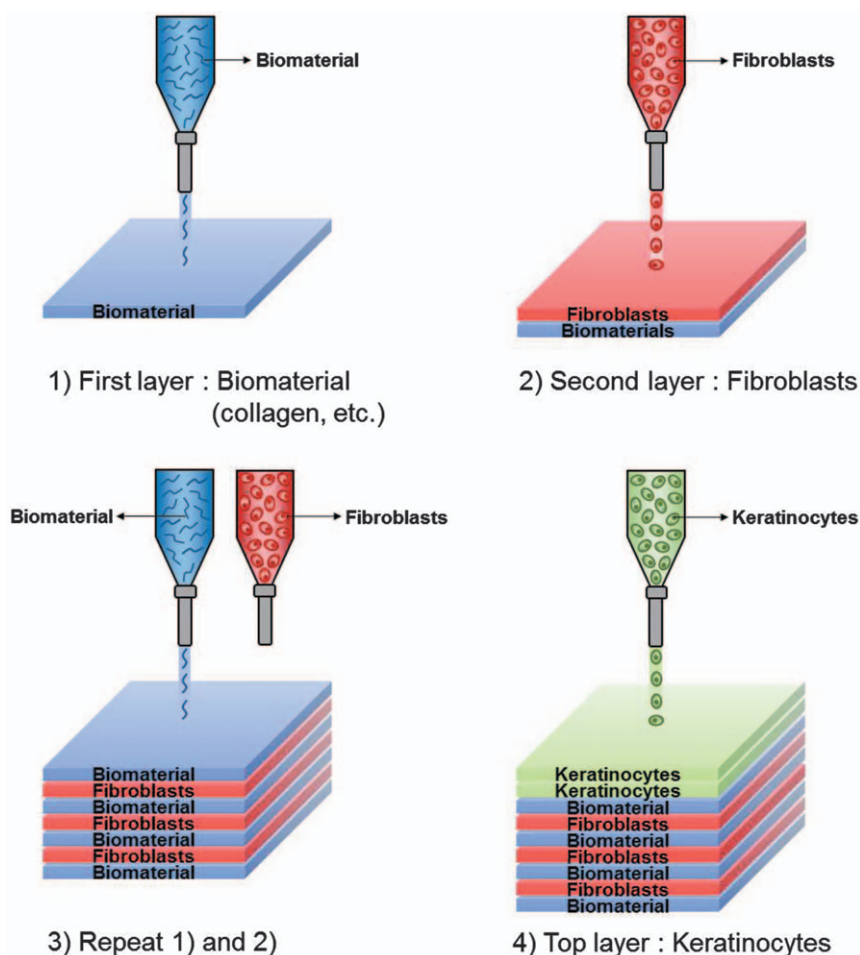


Figure 11.3 Schematic diagram of layer-by-layer printing of three-dimensional (3D) skin tissue.

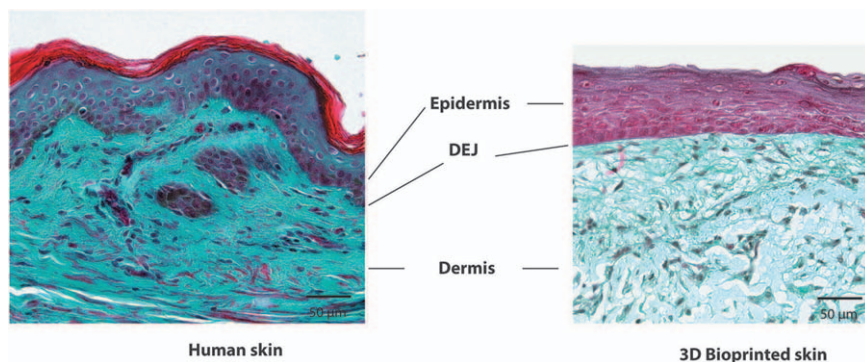


Figure 11.4 Histological microscopy images of human normal skin and 3D bioprinted skin after 26 days of culture. Tissues were stained with Masson's Trichrome.

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applications.²⁵ 3D-bioprinted dermal regions are composed of fibroblasts within a hydrogel matrix. The dermal region is produced by repeated accumulation of fibroblast layers.²⁶ Then, a thin epidermal region can be produced on the dermal region by laminating keratinocyte-laden bio-ink (Figure 11.4). The bi-layered structure can be used to regenerate an artificial skin graft. To enhance tissue regeneration, growth factors are frequently included in bioprinting-based skin tissue engineering.

11.2.5 3D Skin Biofabrication

Hydrogel-based bio-ink usually has poor mechanical properties, which makes it difficult to layer the material 3D printing studies.²⁷ Various kinds of approaches have been introduced to solve this difficulty, such as enhancing the mechanical properties of the bio-ink and improving the printing process, as shown in Table 11.1.^{21,26,28} Lee *et al.* introduced a modulation method for crosslinking bio-ink to produce biomimetic artificial skin.²⁶ They used aerosolized or nebulized NaHCO_3 as the collagen cross-linker and showed that the mechanical properties of the collagen could be modulated by controlling the density of NaHCO_3 . It is not easy to achieve a homogeneous gelation in thermal curing processes due to the heat transfer gradient.²⁶ The cross-linker, NaHCO_3 , ensured homogeneous gelation during the printing process. The authors designed and printed an artificial skin graft with a multilayered cellular construct. The epidermal layer was then exposed to air to induce an air-liquid interface for maturation and stratification. The authors showed that this process formed a stratum corneum.

Koch *et al.* used laser printing technology to print a well-defined skin graft with a composite of blood plasma and alginate hydrogel, containing skin cells such as fibroblasts and keratinocytes.²⁹ They demonstrated enhanced

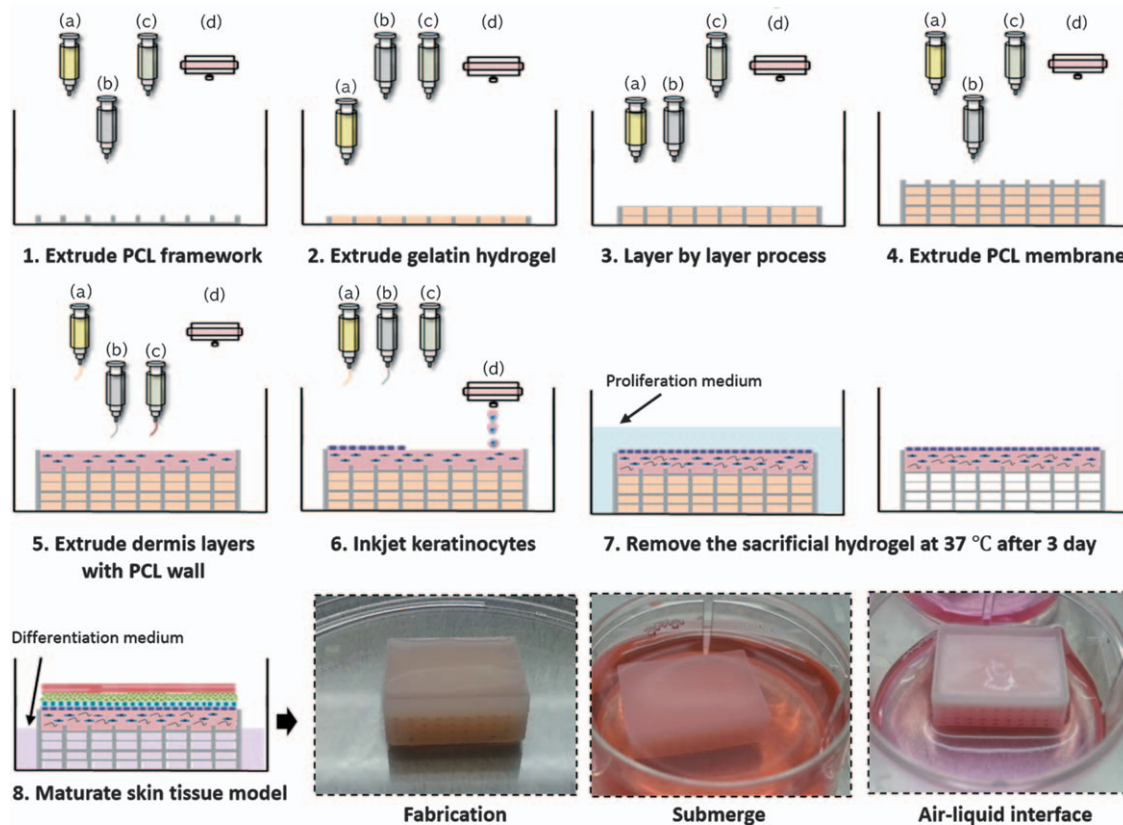
printability by producing a homogeneous layer with a composite material that was suitable for skin tissue engineering applications. They also suggested that this technology could be applied to mesenchymal stem cell-based cell therapy.

Kim *et al.* introduced a co-extrusion process with two hydrogel materials to produce a natural ECM-like cellular construct.²¹ Dissolved collagen and alginate were co-extruded to make a dermal substitute for skin grafts. Collagen has weak mechanical properties and is not appropriate for precisely modulating pore architecture when composing a scaffold.²⁷ To overcome this difficulty, the authors included alginate to enhance the mechanical properties of collagen. They printed a collagen hydrogel construct with a core shell shape, and then created an inner layer using alginate. This composite structure had good mechanical and biological properties. In addition, they were able to control the pore architecture of the printed scaffold. The printed structure was applied in an animal study. The results of this study showed that the printed construct could induce the formation of granulation tissue, epithelization, and rapid vascularization in skin tissue engineering applications.

Yeong *et al.* improved the viability of fibroblasts using a polyelectrolyte gelatin-chitosan hydrogel in a 3D bioprinter.²⁸ Gelatin is well known to be a good biocompatible material,³⁰ and chitosan has been highlighted in studies of wound healing due to its antimicrobial properties.^{31–33} The collagen material has poor printability and a long crosslinking time. The proposed material had a suitable viscosity and good printability, so high-fidelity shapes could be printed using this technology. In addition, the antimicrobial activity contributed to the barrier function of the printed artificial skin.

3D bioprinting technology has also been applied to produce artificial skin models. Kim *et al.* improved the hydrophilic properties of 3D collagen scaffolds using a cryogenic direct-plotting system, and demonstrated that it can be used to regenerate skin.³⁴ After printing, they co-cultured keratinocytes and fibroblasts, and confirmed the migration and differentiation of both types of cells in the scaffold. Conventional natural polymers such as collagen and alginate have hydrophilic properties; this makes it difficult to print 3D structures as it hinders the extrusion of polymers. To overcome this shortcoming, the researchers developed a cryogenic plotting method. The process was realized using a combination of 3D plotting and cryogenic refrigeration. The collagen micro-strands were directly extruded on the cryogenic stage, then lyophilized. This method can produce more sophisticatedly designed 3D scaffolds than other methods, in terms of control over the pore architecture.

Kim *et al.* developed a direct cell printing method for the fabrication and maturation of 3D human skin models in a single-step process.³⁵ The properties of the resulting stabilized skin model included a fibroblast-stretched dermis layer and stratified epidermis; otherwise, it was similar to human skin. Direct cell printing was combined with extrusion- and inkjet-based dispensing modules; the modules were used concurrently to fabricate a porous transwell system and keratinocytes on the dermis layer, respectively (Figure 11.5). The porous transwell system was printed with polycaprolactone



*The lower two heads ("A and B" or "B and C") are the heads that were used in alternating fashion.

Figure 11.5 One-step process to fabricate human skin models with a functional transwell system. A, B, C, D indicate the materials in the extrusion head: (a) sacrificial material (gelatin), (b) supporting material (PCL), (c) bio-ink with cells, and (d) medium with cells. Reproduced from ref. 35 with permission from IOP Publishing, Copyright 2017.

and could mature to a skin model without the use of additives, unlike conventional methods. The produced skin model was stable over the long-term and had an improved tissue morphology, in terms of consolidated differentiation between skin cells.

Most existing bioprinting-based skin wound treatment methods have two-steps: *in vitro* fabrication and transplantation. Binder *et al.* introduced a single-step 3D skin printing technology, so-called *in situ* skin bioprinting, which delivers skin cells directly to the body.³⁶ They developed a new system to realize the single process. Skin cells including fibroblasts and keratinocytes were printed directly onto a bi-layered structure on a full-thickness nude mouse and porcine defect model. Their results showed that wounds closed 3 weeks earlier than other groups. The *in situ* skin bioprinter was composed of two modules: a laser scanning module and cell printing module. The laser scanner was used to obtain information about the anatomical geometry of the full thickness of the animal wound. Based on this data, the printing system generated printing information and delivered fibroblasts or keratinocyte-laden bio-ink directly to the wound site. In another study, the researchers added a growth factor to enhance wound healing. Skardal *et al.* used heparin-conjugated hyaluronic acid hydrogel with an *in situ* bioprinting system.³⁷ The purpose of the hydrogel, a so-called tunable hydrogel system, was long-term release of cytokine and growth factor secreted from AFSCs. They showed that the conjugated heparin can regulate the release time of the proteins. In conventional methods, the cytokines and growth factors are mixed with hydrogel for delivery. This approach is very simple. However, the method is limited by the cost of isolated cytokine, and the actual dynamics of the wound healing phase tend to be complex. Instead, the authors loaded AFSCs into the hydrogel and then printed it to achieve long-term *in vivo* cytokine and growth factor release. The results showed improved wound healing, neovascularization, and the formation of an ECM (Figure 11.6).

Xiong *et al.* produced scaffolds using 3D printing technology with a gelatin-sulfonated silk-fibroin composite containing fibroblast growth factor 2 (FGF-2). They achieved improved epidermal growth and dermal vascularization.³⁸ Silk material has good biocompatibility, suitable mechanical properties, and a non-inflammatory response;^{39–45} in particular, sulfonated silk fibroin supports adhesion, spreading, and the growth of fibroblasts.^{46,47} They conjugated FGF-2 into sulfonic acid to achieve continuous, long-term release. Their animal study demonstrated significantly improved skin regeneration.

11.3 Vascularized Skin Regeneration

Vascular networks deliver nutrients and oxygen and take metabolic waste from tissue.⁴⁸ Hence, these vascular networks are vital to most living cells. There are two strategies to produce artificial vascular networks: cell- and scaffold-based strategies.⁴⁹ Cell-based strategies can be divided into two

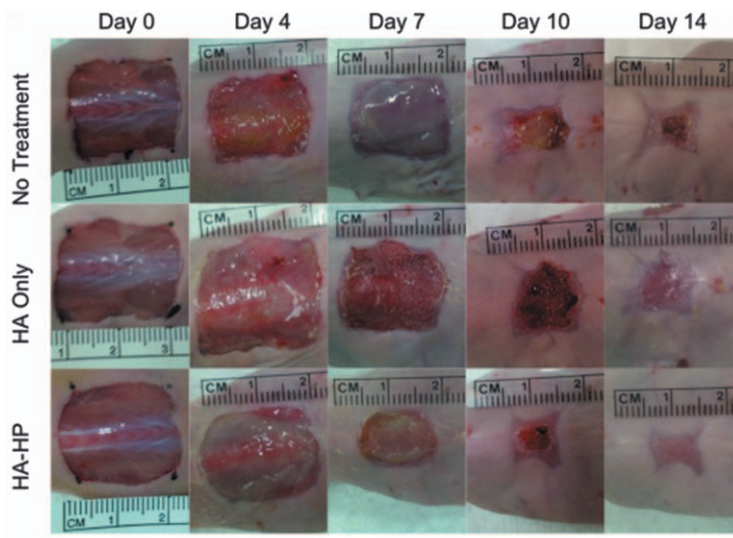


Figure 11.6 Gross morphological images of skin wounds over time show that HA-HP hydrogel accelerates wound closure more than the other groups. Reproduced from ref. 37 with permission from John Wiley and Sons, Copyright 2016 Wiley Periodicals, Inc.

approaches: prevascularization and neoangiogenesis. In prevascularization, endothelial cells with optional supplementary cells such as myoblasts or fibroblasts form vascular networks directly. In neoangiogenesis, cell sprouting is induced from a host blood vessel. Scaffold-based strategies can also be divided into two approaches: one using a microvascular network obtained from a decellularized structure of mammalian skin samples and the other that uses a synthetic scaffold to mimic the tubular structure of blood vessels.

Zhu *et al.* introduced a prevascularized tissue produced by a digital light processing (DLP)-based bioprinting method, referred to as microscale continuous optical bioprinting (μ COB).⁴⁸ A photopolymer including human umbilical vein endothelial cells (HUVECs) and 10T1/2 cells was loaded onto the printer. The cell suspension was then exposed to hexagonally patterned ultraviolet (UV) light to produce cell-laden vascular channels (Figure 11.7). The authors then implanted the printed cellular construct into the dorsal skin of immunodeficient mice, resulting in the formation of a lumen-like structure. Anastomosis with host blood vessels from the host tissue occurred *via* the printed structure.

This kind of vascular structure was used in skin grafts to enhance their functionality. Yanez *et al.* printed vascular layers between skin layers.⁵⁰ The vascular layers were printed on the fibrinogen layer with endothelial cells and thrombin solution. The result was then implanted onto sites of a dorsal mouse wound. A reduced contraction of 17% was observed, compared with the groups who received only skin cells or no treatment. The wounds also

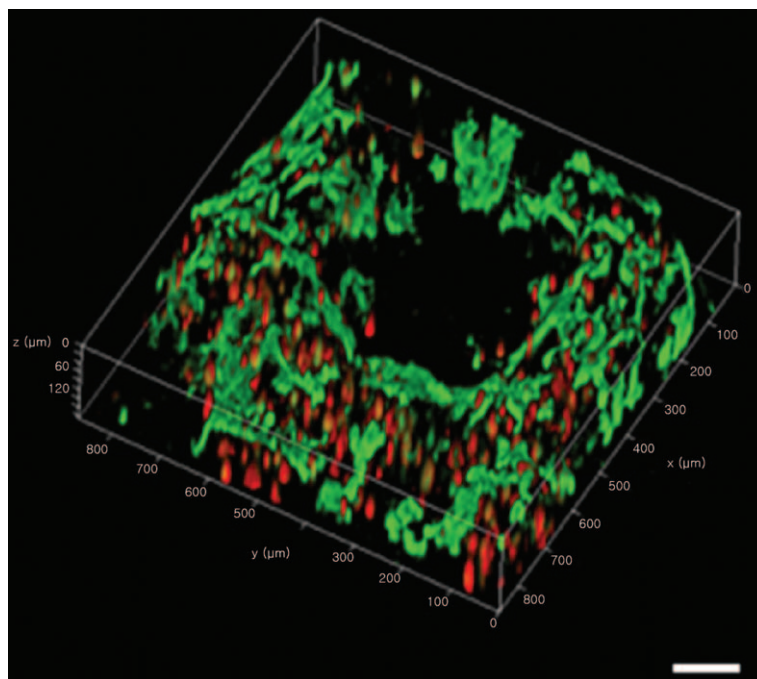


Figure 11.7 3D confocal microscopy image of the printed microchannel walls with endothelial cells. Fluorescent cell tracker (red)-labeled endothelial cells and CD31 (green)-stained endothelial cells (scale bars: 100 μm). Reproduced from ref. 48 with permission from Elsevier, Copyright 2017.

healed faster. The wounds healed and closed fully 5 days earlier in the group with the vascularized graft. This means that vascular networks contribute to skin regeneration.

11.4 Bioprinting of Functional Artificial Skin

Human skin has various kinds of appendages such as sweat glands, melanocytes, and hair follicles. In the case of sweat glands, these are developed from the embryonic epidermis and secrete sweat to maintain body temperature and excrete waste products. Melanocytes are melanin-producing cells located in the bottom layer of the skin epidermis. Melanin is a pigment that forms skin color or hair follicles. It also acts as a sunscreen, protecting the skin from UV radiation.⁵¹ The hair follicles are essential for hair regeneration, and hair protects the skin by preventing the inflow of harmful external materials into the body.⁵²

At present, several researchers have been trying to use bioprinting technology to develop advanced artificial skins that include all the appendages. The main purpose of these studies is to regenerate skin-like layered

morphologies with similar functionality to native skin. Transplantation of the bioprinted functional graft is expected to be more effective than transplantation of simple bi-layered epidermis and dermis structures *in vivo*. These functional grafts can also be used in toxicology tests for new drug candidates and cosmetics.

In 2015, Huang *et al.* introduced 3D bioprinted ECM mimics to facilitate the differentiation of epithelial progenitor cells for regeneration of sweat glands.²⁰ The cells are related to the specification of sweat glands. Epidermal growth factor, an important regulator in the development of sweat glands, was also applied. The components were mixed with a composite of gelatin and sodium alginate and used to print 3D ECM mimics. An *in vivo* examination confirmed that the developed mimics could be used to regenerate sweat glands. Through their continued study, they also published a result indicating self-organizing morphogenesis of sweat glands with bioprinted matrices with precise pore architectures in 2016 (Figure 11.8).⁵³ The porous structure facilitated the self-organization of sweat glands by providing a suitable micro-environment. In this research, several factors including living epithelial progenitors and bone morphogenic protein-4 (BMP-4) were incorporated into the structure during the printing procedure. These were required for effective induction of sweat gland differentiation.

Skin pigmentation has also been studied *via* bioprinting. Min *et al.* introduced melanocyte-laden full-thickness biomimetic skin models with a melanocyte area and spot model (Figure 11.9).⁵⁴ Dendrite formation with melanocytes was observed during culturing of the printed construct. This indicates their survival, and tissue formation. Finally, they successfully achieved visible pigmentation at the boundary region between the fibroblast and keratinocyte layers, which is similar to the organization of natural skin. However, the study only produced the form of freckles or spots. In other words, the printed construct did not reflect the uniform coloring of healthy human skin. These results could be interpreted as consequences of the complex interaction between cells and the non-uniform production of melanin.^{55,56} Although these results cannot fully replicate natural skin pigmentation, they are expected to provide a useful model for skin blemishes such as freckles or ephelides.

11.5 Discussion

There are several difficulties concerning the conventional treatments of skin wounds. Current therapies are usually only suitable for healing superficial wounds, and the transplanted skin does not assimilate with the surrounding tissue. Skin bioprinting technology has been applied to produce advanced functional skin grafts. In particular, 3D bioprinting is an optimum technique for fabricating skin tissue models with the intended 3D geometry through an automatic procedure.¹⁰ However, current 3D bioprinting technology does not achieve the ultimate goals of skin wound therapy.

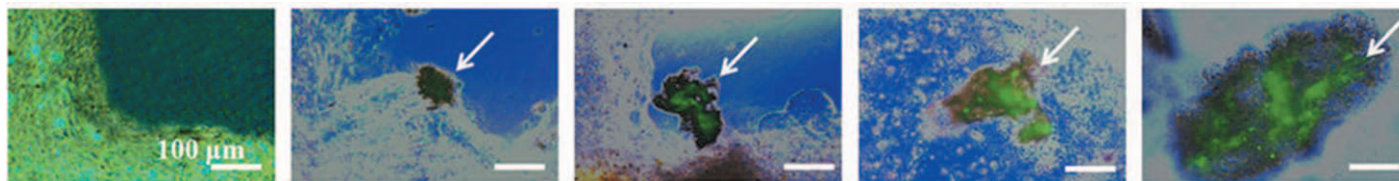


Figure 11.8 Fluorescence images of sweat gland morphogenesis in a sample with a 300 μm nozzle and a plantar dermis construct. The images, from left to right, were taken at the following times: 0 h, day 5, day 14, day 21, day 28 after the culture of epidermal progenitor cells embedded in 3D-printed constructs (scale bars : 100 μm).⁵³ Reproduced from ref. 53 under the terms of the CC BY 4.0 licence, <https://creativecommons.org/licenses/by/4.0/>, <http://dx.doi.org/10.1038/srep34410>, © The Authors 2016.

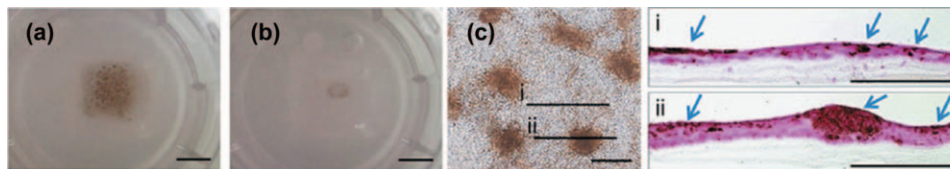


Figure 11.9 Histological examination of biomimetic skin containing melanocyte (MC): (a) MC area model, (b) MC spot model, and (c) distribution of pigmentation with (i, ii) histological cross-sectional views of the MC area model. Reprinted from ref. 54 with permission from John Wiley & Sons, Copyright 2017 John Wiley & Sons A/S.

Natural and synthetic polymers such as alginate, collagen, polycaprolactone, and polylactide are frequently used to construct artificial skin grafts. These materials have long been used in various areas including clinics and regenerative medicine, and has been beneficial in clinical contexts as the safety of the materials has already been verified. However, these materials were developed for bioprinting applications, so their features are not usually suitable as they do not allow for the precise delivery of living cells, 3D patterning processes, and so on. Kim *et al.* improved the mechanical properties and printability by mixing these materials with other materials.²¹ Lee and Dai introduced a decellularization-based bio-ink containing an ECM component with natural tissue to improve the biological effects.⁵⁷ However further study is required to achieve not only good printability but also effective regeneration of artificial skin.

In terms of cell patterning in 3D space, the highest resolution of current 3D bioprinting technology is about 100 μm .¹⁵ This resolution is not sufficient to imitate the microstructure of native skin tissue in detail.⁵⁸ The micro-inner-architecture is closely related to the cellular response to tissue regeneration.²⁴ Therefore, the printing resolution must be enhanced to achieve the ultimate goals of skin tissue engineering. To make the artificial skin economically feasible, the bioprinting procedure must be accelerated. A simple calculation based on current processes indicates that about 3–4 weeks are required to produce therapeutically useful skin with an area of 1 m^2 .¹⁰ This is one of the big obstacles to the application of bioprinting technology in clinics.

Studies into skin regeneration have usually focused on making a simplified bi-layered structure, consisting of an epidermis layer and a dermis layer. Although several researchers have introduced new methods to produce advanced and functional skin substitutes with appendages such as sweat glands⁵³ or melanocytes,⁵⁴ these studies are still in their initial stages. Moreover, to date, there has been no attempt to produce all of these appendages together.

There are several other points to make regarding skin bioprinting techniques. Additional comprehensive studies are required to achieve fully functional skin substitutes. The substitutes should be useable in clinics and for the development of new drugs and cosmetics. The substitutes can be used to treat various kinds of skin wounds including burns, contusions, abrasions, and missing tissue, and are also useful in plastic surgery. Fully functional skin substitutes can be used to evaluate the safety and effectiveness of drugs or cosmetic candidates.⁵⁹ Animal studies are the current gold standard in the development of new products. However, there are numerous well-known issues concerning animal studies, such as ethical problems, high cost, unreliable results and so on. An advanced and fully functional skin substitute will provide a new gold standard that overcomes these issues. Although skin bioprinting still has some difficulties, several researchers have already shown the possibility of regenerating fully functional skin using bioprinting. Continued studies should provide additional information to advance this technology in the near future.

11.6 Conclusion

The skin, which serves as the first line of defense at the outermost region of our body, is one of the most vulnerable organs. Because it is constantly directly exposed to the external environment, such as to pathogens or UV rays, it is often injured by contusion, abrasion, burns, *etc.* Current treatments of extensive and deep skin wounds are still not sufficient to enable full recovery to the skin's original form and function. There are new patients every day, with a huge corresponding social cost. However, current treatments using autografts, allografts, wound dressings, and cell sheets, do not provide enough solutions for skin wound patients. Skin bioprinting is a promising alternative to these, as it can be used to manufacture functional skin substitutes. Numerous studies have been conducted to produce these substitutes. Researchers have designed novel methods for precise bioprinting and achieved enhancements in biological effects. Recent studies have shown that advanced and functional skins with various kinds of skin appendages can be produced using bioprinting techniques. Further studies into skin bioprinting technology will provide novel methods to fully treat skin wounds in the near future.

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CHAPTER 12

*3D Modeling of Hepatic Tissue*MARY C. REGIER^{a,c} AND KELLY R. STEVENS^{*a,b,c}

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12.1 Introduction

The liver is the largest visceral organ and performs a host of vital functions. It is the site of metabolic processes, nutrient synthesis and storage, regulatory activities, and pathogen and xenobiotic defense processes. Owing to the essential nature of many of the liver's functions, liver disease and injury represent significant health concerns that can severely impact quality of life and can result in loss of life. Unfortunately, liver disease remains pervasive globally. For example, viral liver infections hepatitis B and hepatitis C together affect ~400 million individuals worldwide.^{1,2} Additionally, it is estimated that non-alcoholic fatty liver disease (NAFLD) affects ~20% of the Western population^{3,4} including ~9.6% of children and adolescents in the US.⁵ If left unchecked, these diseases and other modes of liver damage can progress to chronic disease states, such as liver cirrhosis and failure. This is a major problem, as one in twenty Americans are estimated to suffer from chronic liver disease.⁶ At the onset of cirrhosis, the

disease becomes curable only through transplantation, and many patients die while waiting for a suitable organ. In 2004, acute and chronic liver disease accounted for a combined 36 000 deaths and ~560 000 years of potential life lost (YPLL, to age 75) in the US. Additionally, liver cancer and viral hepatitis accounted for ~6300 and ~5000 deaths and more than 72 000 and 100 000 YPLL, respectively.⁷

Models that capture liver physiology are key to discovering causes, mechanisms, and treatments for liver disease. Treatments for liver disease would also benefit from systems that capture native liver physiology and pathophysiology or that predict hepatotoxicity. Most preclinical screens of potentially hepatotoxic compounds or studies that probe disease mechanisms have been performed *in vivo* in animal models or *in vitro* using primary human hepatocytes in 2D monolayers. However, many issues related to ethics and predictive abilities plague animal models. Conversely, 2D hepatic cultures have limited liver functions after prolonged culture. Recent developments in the biofabrication of 3D hepatic models demonstrate the promise of novel human-cell-based systems for improving model concordance with *in vivo* liver physiology. This chapter covers the utility and shortcomings of traditional *in vivo* and *in vitro* liver models and more recent results for models produced with 3D structure.

12.2 The Need for Novel Hepatic Models

Detecting hepatotoxicity – The liver is positioned between the absorptive surface in the gastrointestinal tract and drug targets throughout the body. Because of its location and role as the primary site of drug metabolism and biotransformation it is frequently involved in adverse drug effects.⁸ This susceptibility to drug-related damage and the importance of proper liver function make hepatotoxicity a primary concern for patients, regulators, and pharmaceutical companies. Drug-induced liver injury (DILI) can be drug-intrinsic or host-dependent (also referred to as idiosyncratic). In many preclinical studies drug-intrinsic, rapidly developing hepatotoxicity is over-predicted (high rate of false positives), whereas slow developing host-dependent liver toxicity is under-predicted (high rate of false negatives).⁹ One example of drug-intrinsic DILI is acetaminophen hepatotoxicity, which has been shown in a multicenter etiology study to be responsible for ~40% of acute liver failure and result in a ~25% mortality rate for patients.¹⁰ On the other hand, idiosyncratic, host-dependent DILI is difficult to predict, occurring in <5% of the individuals exposed to a drug without clear dose relationships, without temporal patterns, and without apparent relation to the drug's pharmacological effect. Idiosyncratic DILI accounts for approximately 11% of cases of acute liver failure with a mortality rate of ~1-in-3.¹¹

12.2.1 Predicting Drug Metabolism and Toxicity

Beyond direct effects of drugs and their metabolites on the liver, the liver is central to drug absorption, distribution, metabolism, and excretion (ADME), which can adversely affect drug activity elsewhere in the body. Modeling drug metabolism and pharmacokinetics (DMPK), safety, and efficacy in preclinical settings is aimed at predicting the metabolic fate of potential new therapies.^{12,13} Despite investment in extensive *in vitro* testing and expensive *in vivo* preclinical and clinical trials, adverse events related to liver metabolism and transport are common causes of drug removal from the market and black box warnings. With an increasing number of compounds to be tested there is a need for more accurate and cost effective model systems.⁹ Challenges to accurate prediction of DMPK include complex interactions between multiple drugs,^{14,15} effects of multiple tissues,^{16,17} and intra- and inter-species variability.¹³ Humanized rodent models, including those engrafted with human hepatocytes^{18,149} or receiving implants of human hepatic constructs,¹⁹ enable *in vivo* testing where human-specific metabolic processing of drugs can be captured. However, these models remain costly and limited in throughput, include significant variability, and include effects from the host physiology.¹⁴ Research is therefore also being directed toward recreating human ADME processes and downstream effects *in vitro* by representing multiple tissues and by including multiple drugs.^{20–22} These systems have given promising results, but due to their relative complexity coupled with shortcomings in capturing human physiology they have not yet been broadly implemented.

12.2.2 Understanding Liver Disease

Altered diet, exposure to drugs and other chemical compounds, lack of exercise, and stress are characteristic of a modern lifestyle and contribute to diseases such as diabetes, obesity, metabolic syndromes, and heart disease. These increasingly widespread diseases frequently result in liver damage or are caused by liver disease.²³ Additionally, hundreds of millions of people worldwide suffer from infectious diseases that affect the liver, including viral hepatitis B (~250 million) and hepatitis C (~115 million), as well as malaria, which is caused by protozoan *Plasmodium* parasites (>250 million).² These infections lack effective vaccines and/or are incompletely curable. All told, diseases of the liver are major health concerns to the global population. Improved liver disease prevention and treatment may be achieved through the aid of better model systems that enable mechanistic studies and development of therapies targeting that target these afflictions. Unfortunately, animal models often have different liver disease etiologies and susceptibilities compared to humans. Further, human cells in traditional monolayer cultures often fail to fully exhibit hepatic functions and susceptibilities to adequately study liver disease.^{24–27} Emerging human liver disease models promise to improve the physiological relevance and function of such studies.

12.3 *In Vivo* Liver Models

Animal models of liver physiology, damage, and disease have yielded a large portion of what is known about liver function and dysfunction. Animal models enable precise, multi-time point sampling, control over genetics and lifestyle variables, and complex cell-matrix, intercellular, tissue-to-tissue, and organism-wide interaction studies.²⁴ The most common animal models of liver damage are surgical bile duct ligation to model cholestatic injury and carbon tetrachloride (CCl₄) treatment to induce toxin-mediated liver fibrosis.²⁵ Other treatments and dietary modifications can be used to induce liver cancer and to model alcoholic liver disease (ALD), NAFLD, and non-alcoholic steatohepatitis (NASH).^{24,25,28} Surgical procedures, treatment with disease inducing compounds, and changes in diet are compatible across many of the genetic backgrounds available in model species. Importantly, different model strains vary in susceptibility to these insults.²⁵ Additionally, genetically modified animal models have been developed to recreate human liver disease and in some cases reflect human disease-linked mutations.^{29,30} Transgenic mouse models are subject to liver injury due to chronic cholestasis, autoimmune liver fibrosis, NAFLD, liver cancer, and metabolic dysfunction.^{24,25,28,31–33} Finally, humanized or chimeric animal models are used increasingly to study human liver infection, liver gene therapy, drug metabolism, and genetic disease. Typically in these models the mouse liver is populated with human hepatic cells or the animal supports an ectopic human-cell-derived liver construct.^{30–32} These systems enable the study of *in vivo* human hepatic tissue responses to injury, disease, and/or therapy.¹⁵⁰

Although animal models of liver disease have proven useful in understanding liver disease and therapies, their impact on human health has been limited by several factors, such as (1) incomplete recapitulation of human disease (including a lack of confounding health issues and treatments), (2) intra- and inter-species differences in disease susceptibilities and progressions, and (3) resource demands of generating and maintaining animal models. For example, studies have shown that animal models have only ~55% concordance with human hepatotoxicity.³⁴ Three primary issues that confound animal hepatotoxicity results include: (1) high doses used in animal studies compared to human exposures; (2) uniformity of animal models compared to the heterogeneous human population; (3) testing of single compounds in animals as opposed to multiple simultaneous toxicant exposures experienced by humans.^{35,36} Thus, forming human health predictions from *in vivo* model results requires controversial assumptions, extrapolations, and uncertainty factors.³⁶ Similar issues plague the use of animal model systems for studying liver disease. For example, HBV and HCV are examples of the many pathogens that exhibit host tropism. Because of their species specificity preclinical studies involving these diseases must be performed in chimpanzees or human liver chimeric mice. Finally, using animal models is expensive, time consuming, and can raise ethical issues.

For these reasons, novel *in vitro* models that recreate essential aspects of liver structure and functions are being investigated. Such models may be used in parallel to *in vivo* models or may in some cases even replace them.

12.4 Cell Sources for *In Vitro* Culture

Cell lines derived from human hepatomas, such as HepG2, Huh7, and HepaRG, are expandable and show reproducible results. However, these cells are characterized by loss of differentiated function.³⁹ Differentiation of the HepaRG line can improve metabolic and transporter functions. These cells nevertheless exhibit reduced sensitivity to hepatotoxic drugs compared to primary hepatocytes.⁴⁰ To address these challenges, protocols have been developed to differentiate human embryonic and induced-pluripotent stem cells (ESCs and iPSCs, respectively) to hepatocyte-like cells.^{41–43} While pluripotent stem cell-derived hepatocytes express many hepatic markers, these cells are more phenotypically similar to fetal hepatocytes than adult liver parenchyma, which limits their utility for drug development and disease modeling studies. Adult and fetal hepatic stem cells and hepatoblasts (bipotential progenitor cells that differentiate toward hepatocytes or biliary epithelial cells) can also be cultured formats and differentiated toward hepatocyte-like cells. However, the physiologic roles and definitions of these cells remain subjects of debate,^{44–46} they are difficult to source, and they require the development of improved methods for expansion and differentiation for *in vitro* applications.³⁹

12.5 2D Hepatocyte Cultures

Due to species-to-species differences in hepatotoxic and liver pathophysiology, much research has sought to utilize primary human hepatocytes to model human liver injury and disease. Isolated hepatocytes in traditional monolayer culture undergo rapid phenotypic changes, including the down-regulation of liver specific enzymes, secreted albumin, and transporters.^{37,38} Media supplementation, high seeding density, and extracellular matrix (ECM) substrate coatings can extend hepatocyte viability and function for a few days, but this does not prevent dedifferentiation.³⁸ Furthermore, utility of primary human hepatocytes has been limited by intrinsic donor variability, limited accessibility, and limited proliferation in culture. As a result of these challenges, the field has explored a variety of alternative cell sources and culture methods.

12.5.1 2D Sandwich Culture

In healthy liver tissue, hepatocytes are organized into plates or cords, in which basal surfaces face the Space of Disse and sinusoid, and apical surfaces form ~1 μm diameter bile canaliculi between adjacent hepatocytes.⁴⁷

Hepatocyte polarity is essential to liver function because it enables clearance of endogenous, xenobiotic, and chemical toxicants from the basolateral (sinusoidal) surface to the apical (biliary) space for excretion. To stimulate polarization, Dunn *et al.* established a collagen “sandwich” configuration for culturing primary hepatocytes in 2D. With this method, hepatocytes are seeded onto one layer of collagen and overlaid with a second layer of collagen, leading to changes in morphology (including bile canaliculi formation) and function.⁴⁸ This initial study showed that culture between layers of collagen type I preserves albumin secretion for 6 weeks, whereas culture over a single layer of collagen results in a complete loss of albumin secretion in ~1 week. The addition of a second, overlaying layer of collagen after seven days of culture on a single gel is sufficient to restore albumin secretion.⁴⁸ Despite improvements in hepatocyte morphology and function relative to standard monolayer culture, collagen sandwich culture results in variable changes in hepatic function compared to freshly isolated primary hepatocytes. For example, apical efflux transporters, basolateral efflux transporters, and metabolic enzyme mRNA levels and protein activities show a steady or sudden decrease, others increase over time, and still others demonstrate biphasic patterns of decrease and increase.⁴⁹ These models are useful nevertheless due to their throughput and ability to recreate some important physiological mechanisms, particularly in modeling biliary drug clearance.¹²

12.5.2 2D Co-culture Models

In addition to cell–matrix and cell–cell interactions between hepatocytes, liver functions are significantly impacted by heterotypic interactions between hepatocytes and other types of cells. For example, hepatotoxicity is frequently dependent on dynamic and reciprocal interactions between multiple cell types (parenchymal and immune, stromal, endothelial, and/or neural cells) rather than on the reaction of hepatocytes alone.³¹ Co-culture of hepatocytes with a variety of cell populations *in vitro* preserves and restores some physiologic hepatic functions^{50–53} and models complex *in vivo* interactions.^{2,27,54–56} Primary hepatocytes and other liver cell types have been co-cultured in 2D monolayers in which both cell populations are randomly organized, in shared media but on physically separated substrates, or patterned across the same substrate.⁵⁷ Cell lines and primary cells derived from other tissues also support hepatocyte functional maintenance in co-culture.^{51,58} Controlling the extent of homotypic and heterotypic interactions through micropatterning of co-cultured cell types has proven to have a pronounced effect on hepatic functional stability and thereby extend major liver functions for weeks of culture.^{58,59} To date several techniques have been developed to establish 2D micropatterned co-cultures.^{58,60–63} Such culture platforms improve *in vitro* prediction of DILI, drug clearance, and drug–drug interactions^{27,64–67} and predicted functional effects of proinflammatory cytokines.⁶⁸ Additionally, micropatterned co-culture systems have also been

designed to identify niches for hepatic stem cell differentiation⁶⁹ and to enable *in vitro* hepatic viral and parasitic infection.^{2,70,71} Finally, co-cultures in microfluidic devices have enabled control over the soluble environment including factors such as oxygen tension, which influences hepatotoxicity,⁷² and paracrine signal exchange, which can determine hepatic precursor fate choices.⁵⁶

12.6 3D Model Systems of the Liver

The liver is a densely populated organ consisting of tessellating lobules with a well-defined 3D structure. Hepatocytes alone exist at a density of 120 000 000 cells g⁻¹ in human liver tissue,⁷³ and are functionally supported by tissue-specific endothelial, stromal, epithelial, and immune cell types. Accordingly, healthy liver is a relatively matrix-poor organ.³¹ Critically, 2D culture limits the extent of cell–cell interactions (*i.e.* single layer of cell junctions), alters the distribution of cell–matrix interactions (*e.g.* large matrix to cell ratios in sandwich cultures), and effects the profiles of factor transport between cells and from the environment (*e.g.* dilution of soluble factors into large volumes of media). Although 2D hepatic cultures differ from native tissue in these respects, they have clearly demonstrated the importance of direct and indirect cellular interactions as well as cell–matrix interactions. Thus, new fabrication methods have been developed to generate *in vitro* models that mimic some aspects of the 3D nature of *in vivo* hepatic tissue.

12.6.1 Hepatic Spheroids

The most straightforward method to fabricate a 3D liver model is the hepatic spheroid. Provided substrate adhesion is prevented, isolated hepatocytes, pluripotent stem cell-derived hepatocyte-like cells, and hepatoma cell lines can be aggregated into self-organized 3D structures.^{74–76} Hepatic spheroids are typically formed in non-adhesive culture dishes or microwells, in agitated culture (rocking, rotating, *etc.*), or in hanging drops.⁷⁷ One comprehensive characterization of primary human hepatocyte spheroids has demonstrated multiple advantages of spheroid culture as compared to *in vivo* liver and in contrast to 2D cultures.⁷⁸ This study showed that spheroid proteomes cluster with *in vivo* tissue and away from 2D cultures. Hepatocytes in spheroids also retain their patient-to-patient variability as indicated by proteomic clustering of spheroids with the corresponding donor tissues.⁷⁸ Further, spheroid culture can stabilize the phenotype of primary human hepatocytes for more than five weeks. Hepatocytes in these cultures retain their morphology, remain viable, and demonstrate hepatic functions over this time period.⁷⁸ This stability enables chronic exposure to hepatotoxic compounds, increasing assay sensitivity and improving the detection of physiologically relevant toxic doses.⁷⁸ Compared to 2D cultures, human hepatic spheroids have demonstrated increased sensitivity to known

hepatotoxicants, similar hepatotoxicity detection specificity, and improved accuracy in pharmacological classification.⁷⁹ As compared to other *in vitro* models, primary human hepatic spheroids have been found to be uniquely capable of predicting the chronic toxicity of fialuridine.⁷⁸ Primary hepatocyte spheroids also exhibit inducible bile acid and lipid accumulation indicating potential applications in modeling cholestatic and steatotic disease.⁷⁸ Finally, spheroid culture of easily-sourced human hepatocarcinoma cell lines has been demonstrated to increase their relevance to normal hepatic physiology. Specifically, spheroid culture increases hepatocyte-specific metabolic, transport/efflux, and synthesis functions and decreases matrix, adhesion, and oncogene transcript expression for hepatocarcinoma cell lines.^{80–82}

Similar to 2D systems, co-cultures of hepatocytes and other non-parenchymal liver cells, cell lines, or cells derived from other tissues in spheroid formats can also increase and prolong hepatocyte-specific function.^{76,83–85} For example, mixed co-culture of primary rat hepatocytes with the rat hepatic stellate cell line HSC-T6 enhances metabolic activity and increases ECM deposition throughout the spheroids, without interfering with the formation of bile canaliculi, tight junctions, and desmosomes.^{84,86} Rat hepatocytes also showed elevated albumin and enzyme expression when cultured in spheroids with HSC-T6 cells.⁸⁷ These results point toward the functional and structural significance of hepatic stellate cell and hepatocyte interactions. When isolated rat or human hepatocytes are recombined with the corresponding non-parenchymal fraction, endothelial cells have been shown to localize to the exterior of the spheroids with hepatic stellate cells and Kupffer cells distributed throughout.^{31,78,82} In another example of heterotypic self-organization, rat hepatic spheroids have been coated with collagen I, which supports seeding of human umbilical vein endothelial cells onto the spheroid exterior. Inoculation of a hollow fiber with these spheroids results in the establishment of a hepatic tissue containing a dense vascular network.⁸⁸

Due to ease of fabrication, compatibility with multi-cell type interactions, and maintenance of hepatic function in spheroid culture, hepatic spheroids are frequently used as a starting configuration in more complex molded, microphysiologic, or bioprinted systems. However, spheroid culture is subject to limitations and concerns. For example, spheroid size is difficult to control using some fabrication methods (e.g. rotating wall vessels), which adversely effects reproducibility. Due to the high metabolic activity and microenvironmental regulation of phenotype, nutrient and oxygen supply is of particular concern in 3D hepatic tissue cultures. For example, spheroid size is of particular importance in terms of maintaining sufficient oxygen supply in spheroid cores. For mouse hepatocyte spheroids in a rotating wall vessel, cores are estimated to reach critically low oxygen concentrations as spheroid diameters surpass $\sim 200\ \mu\text{m}$.⁸⁹ While the relatively small number of cells per spheroid may increase throughput, their size and variability is likely to result in the need for a number of samples to acquire consistent quantitative measures.¹²

Several platforms have been developed that use microfabrication approaches to precisely control hepatocyte spheroid size, encapsulation, and co-culture with other cells. Adjusting microwell size and cell number per well are straightforward and effective methods for controlling spheroid size.^{76,85} Cell number per aggregate has been shown to significantly impact hepatocyte function with *in vitro* and after *in vivo* implantation. In both environments spheroids that are either too large or too small support decreased albumin production compared to the optimized ~100 hepatocyte/microwell condition.⁷⁶ As with spheroids formed on non-adhesive surfaces, hepatocytes microfluidically encapsulated in PEG or alginate shells or embedded in alginate-collagen microgels show increased albumin and urea production compared to 2D controls.^{90–92} Additionally hepatic spheroids formed in microfluidically generated PEG capsules are more uniform in size and circularity as compared to spheroids formed in non-adhesive microwells.⁹⁰ Co-culture with fibroblasts in a monolayer underlying spheroid capsules,⁹⁰ fibroblasts embedded in the capsule shell,⁹¹ or fibroblasts co-embedded in microgels⁹² further increases hepatocyte-specific synthetic function. Rather than spherical capsules, microfluidic patterning of hepatocytes sandwiched between NIH-3T3 streams enables the encapsulation in alginate microfiber constructs, mimicking hepatic cords. These cords support increased albumin secretion, urea synthesis, and hepatic gene expression over >40 days of culture as compared to 2D cultures and mono-culture fibers.⁹³ Finally, in an alternate approach, microelectrodes have been used to pattern the encapsulation of hepatocytes into liver lobule inspired, six sided disks. These microtissues outperform similarly sized spheroids in albumin and urea production.⁹⁴ These methods of 3D cell-interaction patterning generate uniform multicellular units that may serve as building blocks for larger or more complex 3D constructs, which may further enhance their *in vivo* relevance.

12.6.2 Liver Organoids

Historically the term ‘organoid’ has been loosely applied to describe any 3D organotypic culture of primary cells, stem cells, or cell lines or in reference to cultured explanted organs and tissues.⁹⁵ More recently, organoids have been defined to be *in vitro* cultures derived from stem cells or progenitor cells which undergo self-guided assembly, are capable of limited self-renewal, and differentiate to exhibit functions characteristic of their tissue of origin.^{96,97} Here, we use the latter, modern definition of organoids. Frequently, liver organoid cultures contain primarily hepatic precursors or progenitors and lack relevant supporting cell types such as hepatic stellate cells, liver sinusoidal endothelial cells, Kupffer cells, and neural cells. Instead, these 3D cultures rely on extracellular matrices (ECM), most commonly Matrigel, and, in some cases, cells derived from other tissues to facilitate their recapitulation of tissue structure and function.^{98,99}

Using an approach similar to that used to derive intestinal organoids,¹⁰⁰ Huch *et al.* produced liver organoids from sorted Lrg5 + hepatic stem cells

isolated from injured mouse livers. With this method cells are embedded in Matrigel in combination with signaling modulation through media supplementation, resulting in subsequent organization into proliferative and polarized cyst structures.¹⁰¹ Huch and coworkers have also formed organoid cultures from EpCAM+ ductal cells from normal human liver tissue, and from biliary duct fragments.^{101,102} These adult tissue derived hepatic organoids are genetically stable, suitable for expansion beyond one year, and compatible with cryopreservation.^{101,102} Using these methods, liver cells cultured as organoids are primarily hepatic progenitors, but can be differentiated toward a more mature hepatocyte phenotype through inhibition of Notch and TGF- β signaling.^{101–103} Importantly, biopsies and cell aspirates are sufficient for the generation of this class of liver organoids.⁹⁸

3D organoid cultures have also been formed by combined seeding of multiple populations of purified primary cells or stem cell-derived cells on layers of Matrigel. Takebe *et al.* demonstrated the potential for human hepatic-specific endoderm iPSCs, HUVECs, and MSCs in direct cell-to-cell contact to self-assemble into 3D embryonic liver bud-like structures under these conditions. These tissues exhibit increased early hepatic marker gene expression similar to embryonic mouse liver buds.⁹⁹ It was recently demonstrated that paracrine factors from HUVECs and MSCs are sufficient to promote increased expression of hepatic genes, but cell-to-cell contact is required for 3D morphogenesis of liver bud-like cultures.¹⁰⁴ Upon implantation into mice, human liver buds that are generated *in vitro* form liver tissue with functional vascular networks.⁹⁹ Primary human cells (hepatocytes, liver sinusoidal endothelial cells, and mesenchymal stem cells), genetically engineered to be transiently proliferative through lentiviral gene delivery, also self-organize on Matrigel. These 3D tissues show enzymatic function, hepatocyte-specific markers, and enhanced *in vivo*-like morphology compared to 2D cultures, but unexpectedly remain proliferative beyond 10 days.¹⁰⁵

A primary benefit of organoid culture is that it enables patient specificity of models and recapitulation of genetics-based liver diseases and therapies. Liver organoids that mimic *in vivo* pathologies have been generated from the tissue of patients with Alagille syndrome and α 1-antitrypsin deficiency¹⁰² and from a canine model of Wilson's disease.¹⁰³ In the case of the canine copper-storage disorder model, lentiviral transduction with a corrective gene is sufficient to restore organoids to the wild-type phenotype. This result demonstrates the feasibility of therapeutic genetic interventions in organoid cultures.¹⁰³ Organoids derived from mouse, human, dog, and cat liver tissue have also been used to investigate inter-species differences in predisposition to lipid accumulation and hepatic steatosis.¹⁰⁶ All told, primary tissue-, stem cell-, and modified primary cell-derived organoids should be valuable resources for studying liver physiology, for modeling liver disease, and for personalized clinical diagnostics and regenerative medicine.⁹⁸ However, both embedded and overlaid organoids require expensive matrix and media supplementation regardless of cell source, which

limits their throughput. Furthermore, self-organization and matrix composition, which frequently relies on Matrigel, is largely uncontrolled in these cultures.

12.6.3 Microphysiological Hepatic Culture Systems

In addition to microscale patterning of a 2D culture, microscale 3D cultures have been developed to improve *in vitro* control and recapitulation of the native liver tissue environment. Such “microphysiological systems” as *in vitro* liver models offer: (1) precise control over cell, matrix, and media positioning; (2) low cell number and reagent amount requirements; and (3) compatibility with perfusion of the culture and combination with other model systems (*i.e.* fluidic connection to other tissue models). For example, microscale patterning of complex cellular interactions¹⁰⁷ has been shown to improve recapitulation of hepatic function *in vitro*. Additionally, culture in microfabricated devices improves hepatic function due to increased accumulation of endogenous signals¹⁰⁸ and spatial confinement.¹⁰⁹ Control over media perfusion in 2D¹¹⁰ and 3D¹¹¹ cultures improves hepatic functions. Furthermore, the cell to blood ratio found *in vivo* (<10 nL blood per 1000 hepatocytes) has been more accurately modeled using small volumes of perfused media in microfluidic systems than in static and more dilute well plate culture (>1 μ L media per 1000 hepatocytes, with abrupt media substitutions every 1–2 days).¹¹²

The ability to enhance media exchange through perfusion in microphysiological systems has enabled improvements in *in vitro* hepatic function and unique *in vivo*-inspired patterns of cellular interactions. Because the majority of the liver's functions pertain to conditioning the blood (*e.g.* removing toxins, synthesizing albumin, and metabolizing xenobiotic compounds), flow-associated exchange of nutrients and other molecules is an essential feature of liver physiology. The benefit of perfusion in arrays of lobule shaped compartments containing induced-pluripotent stem cell-derived hepatocytes has been demonstrated by increased production of albumin and urea compared to static cultures.¹¹³ Microscale bioreactors featuring the direct perfusion of 3D hepatic cultures seeded in microwells on a permeable membrane enables self-organization,¹¹⁴ increased synthesis of albumin and urea,¹¹⁵ and maintenance of metabolic activity¹¹⁶ in response to flow. The inclusion of Kupffer cells in pneumatic micro-pump driven embodiments¹¹⁷ of this technology enables functional responses to inflammatory and anti-inflammatory conditions. For example, cultures display cytokine secretion in the presence of lipopolysaccharide,¹¹⁸ *in vivo*-like (23.8 l h^{-1} *in vitro* versus 18 l h^{-1} *in vivo*) clearance of anti-inflammatory hydrocortisone,¹¹⁸ and modulation of hepatocyte cytochrome P450 activities with two weeks of IL-6 inflammatory stimulus.²¹ Perfused 2D hepatocyte cultures have also demonstrated drug metabolic activity similar to *in vivo* values, but have been more limited in culture duration.^{119,120} In another system, radial flow is established from six peripheral inlets across a hepatic

culture (initially 2D) to a central outlet.¹²¹ This flow pattern is designed to represent that of the liver lobule, where blood is supplied through six pairs of portal veins and hepatic arteries and exits through the central vein. Cultures subjected to radial flow form architectures similar to *in vivo* hepatic cords. After 7 days of culture, static cultures are desensitized to acetaminophen toxicity whereas radially perfused cultures demonstrate zonal sensitivity.¹²¹

In vivo, the liver is perfused with blood containing nutrients, signals, waste products, and toxins through the network of liver sinusoids. The liver sinusoid is a unique vessel in that it is lined with specialized, fenestrated endothelial cells. Fenestrae are 0.1–0.3 μm diameter openings in the endothelial lining that allow free diffusion of molecules from the plasma to the basal surface of hepatocytes in the Space of Disse while reducing shear stress on hepatocytes.^{122,123} Modeling the liver sinusoid therefore has important implications in predicting drug metabolism and drug-related toxicity and in recreating normal and aberrant liver physiologies. The influence of indirect flow has been demonstrated in a sandwich configuration where rat hepatocytes are separated from a media flow channel by a layer of collagen type I and a transwell membrane. The cells are protected from fluid shear but are subject to increased solute exchange with the media. In this context hepatocytes form a more continuous monolayer with interspersed bile canaliculi, demonstrate elevated synthetic function, deposit more type IV collagen, and are more enzymatically active compared to hepatocytes in static culture.¹²⁴ The fenestrated endothelial layer has also been modeled simply *in vitro* using microscale channels or gaps between micro-posts located between a central channel containing densely seeded hepatocytes and a channel containing media flow.^{111,125–128}

More cellularly complex systems have been used to model the sinusoid in stacked 2D culture using permeable membranes seeded with endothelial cells positioned over a hepatocyte-containing compartment.^{109,129} Several recent models have included Kupffer cells co-seeded with the endothelial layer and hepatic stellate cells in the hepatocyte compartment¹⁰⁷ or substitution with cell line analogues.^{130,131} This configuration mimics cell distribution on either side of the Space of Disse. Though inflammatory modulation of static transwell cultures featuring this four cell type configuration has elicited changes in hepatic functions,¹³² the introduction of flow in the vascular compartment has been shown to significantly affect both compartments. For example, flow over a liver endothelial and Kupffer cell layer either induced or increased HGF secretion from non-parenchymal cells while albumin production and enzymatic activity are increased in the hepatocyte compartment.¹⁰⁷ Perfusion through the endothelialized channel has also been shown to increase microvilli formation, synthetic activity, and metabolic function in the hepatic compartment.¹³¹ In a unique demonstration of functional response to inflammatory signals the co-culture featuring all four cell types supports increased neutrophil adhesion than endothelial cells alone or endothelial–Kupffer cell co-cultures.¹⁰⁷ Finally, 3D

perfusion models of endothelialized hepatic tissues with hierarchical vessel networks demonstrate drug clearance and have increased urea synthesis compared to a 2D sandwich culture.¹³³ Models consisting of liver lobule-like structures show enhanced effects of drug–drug interactions and higher enzymatic activity compared to non-perfused 3D mono-cultures.¹³⁴

12.6.4 Integrated Microphysiological Systems

In addition to being directly affected by hepatotoxic compounds, the liver plays other essential roles in determining both desired and adverse drug effects. Integration of liver-on-chip modules with cultures representing other tissues together in body-on-chip platforms (also termed micro-cell culture analogues, μ CCAs) has been investigated as a means to predict drug metabolism, drug-induced toxicity involving other tissues, and drug efficacy. Many pioneering and present day microfluidic multi-tissue models have featured compartments with 2D surfaces^{17,135,136} or carrier beads¹³⁷ seeded with hepatic cells. These cultures have demonstrated multi-tissue dependent drug absorption, metabolic processes, toxicity responses, compound bio-distribution patterns, and drug bioactivity. Increasingly, compartments in interconnected tissues-on-chip contain 3D tissue models. Such systems have enabled the demonstration of interactions between liver and other tissue analogues as well as multi-tissue drug interactions. Microfluidically connected co-cultures of neurospheres and hepatic spheroids are more sensitive and dose-responsive than single tissue models to neurotoxin indicating potential utility in toxin detection. Similarly, hepatic spheroids are capable of metabolic bio-activation of drug that slows the growth of tumor spheroids in connected co-culture.¹³⁸ Thereby, this μ CCA can detect downstream implications of metabolic drug modification in liver tissue. More complex models of tissue interactions will require substantial *in vitro* optimization and *in vivo* validation. Liver-gut cross-talk in the context of inflammation has been modeled through the co-culture of perfused, 3D hepatocyte–Kupffer cell microphysiological systems with transwell-cultured intestinal cell lines. Under inflammatory stimulus connected liver and gut analogue responses can be described as additive, subadditive, and synergistic in terms of cytokine expression while showing reduced tissue-specific activity.¹³⁹ How these results relate to inflammation responses of the tissues *in vivo* remains to be demonstrated. However, these experiments have provided testable hypotheses.

μ CCAs commonly illustrate tradeoffs between increased functionality and increased cost or difficulty of use. Although device complexity has hampered the broad utilization of specialized μ CCAs, in some cases, device design can eliminate or mitigate issues faced in less customized model systems. A primary concern when performing co-cultures of multiple cell types is media compatibility across all cell types, whereas systems with barrier tissue equivalents (e.g. intestinal or vascular barriers) present the opportunity for the partitioning of media formulations. In one system, a liver

spheroid-containing chamber has been integrated with intestine, skin, and kidney proximal tubule barrier equivalents and three media are used with two flow rates. With different media supplementation in the apical intestinal compartment, the blood-equivalent compartment, and the excretory side of the proximal tubule epithelial compartment all tissues remain viable, transcriptionally stable, and well-structured over four weeks of culture.¹⁴⁰ This case illustrates how the benefits of added complexity may offset associated drawbacks.

Due to the vast range of possible model configurations and the gains made through *in vivo*-inspired cultures, the development of μ CCAs has focused on faithful recapitulation of structure and proportions of the human body. Thus, device design has been aimed at maintaining proper scale for media to cell ratios, physiological shear, and appropriate media residence times across each tissue compartment. Microscale culture chambers and the connecting fluidic channels in some devices have therefore been designed to support appropriate volumes and flow rates. For example, a 14-compartment device was designed such that it contains four barrier tissue compartments and ten solid tissue compartments. Five tissues – lung, intestine, liver, bone marrow, and kidney – have been represented by cell lines cultured across transwells (lung and intestine) or suspended in matrix (liver, bone marrow, and kidney). These tissue equivalents have been perfused with physiologically relevant flow rates through the tuning of channel geometries and gravity-driven flow. Cells in each compartment remain viable and liver tissue equivalents are synthetically and enzymatically active over seven days of culture.²⁰ However, with its necessary reservoirs this device maintains a fluid to cell ratio five-fold higher than the average *in vivo* circulating fluid to cell mass ratio. Because certain aspects of human physiology are difficult to scale together, μ CCA design will continue to work toward translating human tissues to the microscale.

12.6.5 Bioprinted Liver Models

Rather than relying on cell driven organization or fluidic patterning, bioprinting of liver tissue enables direct “printing” of cells in matrix. Bioprinting is generally divided into two categories: stereolithographic cross-linking of cell-containing matrix (“photopatterning”), or the extrusion-based direct, additive deposition of cells in matrix. These approaches theoretically enable complex architectures to be generated, such as vascular trees and patterned stromal-parenchyma interactions. For example, hepatocyte-fibroblast co-cultures within hexagonal lattice structures generated by stereolithographic photopatterning have shown improved synthetic function relative to unpatterned controls.¹⁴¹ Hybrid 3D cultures consisting of two interlaced patterns of different material formulations containing iPSC-derived hepatocytes or endothelial cells and adipose-derived stem cells have been designed to recreate the structure of arrayed liver lobules. These constructs support increases in albumin and urea synthesis and cytochrome

P450 enzyme gene expression.¹⁴² In general, stereolithographic bioprinting has the potential for rapid layer-by-layer fabrication, but is limited by the number of photo-crosslinkable matrix materials available.

Extrusion-based bioprinting has also been used to print hepatic tissues using multiple cell types in free standing cultures.^{143,144} This method has also been combined with microfluidic devices to enable perfusion. For example, 3D printed tissues have been pre-printed and placed in microfluidic devices,^{145,146} printed *in situ*,¹⁴⁷ or printed in parallel with microfluidic device housings.¹⁴⁸ These cultures have demonstrated detection of hepatotoxicity, maintenance of stem cell differentiation potential, and drug metabolism. For example, preformed hepatic spheroids printed into a microfluidic bioreactor show sustained hepatic function and sensitivity to acetaminophen.¹⁴⁷ Because of their rapidly tunable structured design, 3D bioprinted hepatic tissues have the potential to be used in settings where adjustment of the construct architecture enables physiological function modulation or affects mechanism of hepatotoxicity.

12.7 Conclusion and Future Perspectives

For 2D and 3D *in vitro* liver model systems to significantly enhance our understanding physiology in healthy and disease states and to impact the safety and efficiency of drug development, several biological and technical improvements will likely still be required. For example, sourcing of relevant human hepatocytes remains problematic. Because of the scarcity of primary human hepatocytes and insufficient quality of pluripotent stem cell-derived hepatocyte-like cells primary hepatocytes from other species (usually rat) remain prevalent in model development. As such, the potential for *in vitro* models to replace physiologically disparate animal models with human-cell-based systems has, in many cases, not been realized. New methods for maturing stem cell-derived hepatic cells or for translating the *in vivo* regenerative capacity of liver tissue to efficient *in vitro* propagation of primary human hepatocytes both represent potentially transformative advances. The use of advanced methods for culture assembly is likely to figure prominently into realizing improved cell sourcing.^{151–153} Rat and other animal-derived hepatocytes will remain useful, particularly in studies directly comparing human- and animal-derived *in vitro* liver models. Such comparisons may help improve capabilities to relate results from *in vivo* animal models to human toxicity and disease.¹⁰⁶

The benefits of 3D models of liver tissue have been attributed to the recreation of features of *in vivo* liver tissue environments. However, it is essential that the added biological and technical complexity of *in vitro* models result in significant and reproducible gains in model performance such that any added costs and complexities are warranted. While some co-culture and structural arrangements have demonstrated the potential for improvement in physiological modeling and toxicity and metabolic effect prediction, many structural and heterotypic features of native tissue remain

to be explored. For example, though the formation of bile canaliculi is commonly reported, no model has included a patent and functional biliary system for bile clearance. Such models will likely require new or modified biofabrication methods. Additionally, owing to the significant role of inflammatory immune response in liver pathologies, integrated microphysiological system featuring representation of the adaptive immune system has the potential to improve a range of model capabilities.¹¹² Other aspects of essential aspects of *in vivo* liver tissues have seen limited or no representation in 3D *in vitro* models, such as innervation.

Finally, model input and configuration improvements should be paired with enhanced readouts. Albumin and urea synthesis rates, cytochrome P450 activities, and metabolism of a subset of compounds are very frequently used as indicators of hepatocyte function. However, these few readouts capture a small portion of the *in vivo* functions of the liver. More omic scale analyses promise to improve the breadth of characterization to more comprehensively assess model relevance.¹³⁹ Transcriptomics should enable ‘snapshot’ comparison of cell states, while secretome characterizations have the potential to be conducted frequently throughout model culture as non-endpoint measures. For microphysiological systems the challenge of sample size may require modified customized measurement approaches¹⁵⁴ or sample pooling. Additionally, correlation between cell morphology and function may also help assess model fidelity throughout culture.

Overall, *in vitro* 3D models have become increasingly common and have shown potential advantages in recapitulating key liver functions. Ultimately, these models must show significant improvements over simpler *in vitro* model systems and compared to more complex *in vivo* approaches. Thus, the field must demonstrate both functionality and practicality to achieve widespread and impactful adoption. Biofabrication of such systems should be therefore be geared toward minimizing cost and effort for operation and maximizing the relevance of results.

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CHAPTER 13

Microphysiological Models of the Respiratory System

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13.1 Introduction

The lung has an anatomically unique structure consisting of a branching network of conducting tubes that enables convective gas transport to and from alveolar compartments where gas exchange occurs. Throughout development and adult life, the respiratory system experiences a variety of physical forces imposed by structural changes in surrounding tissue, continuous passage of fluids, and cyclic mechanical deformation of the basement membrane and extracellular matrix. As the most essential cellular constituent of the respiratory system, the epithelial cells comprising the luminal surface of airways and alveoli are known to sense and respond to this dynamic mechanical environment. During fetal lung development, mechanical forces generated by airway distension and intermittent fetal breathing movements have been shown to profoundly influence the proliferation, apoptosis, and differentiation of pulmonary epithelial cells.^{1,2} Mechanical perturbations in the mature lung also play a critical role in regulating the structure, function, and metabolism of the epithelial cells.³ Unusual changes in the mechanical environment of the respiratory system often contribute to the progression and exacerbation of various pulmonary disorders by eliciting abnormal biological responses of epithelial tissue in airways and alveoli. For example, large deformations of the alveolar

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basement membrane due to overdistension of the lung during mechanical ventilation can induce injury of alveolar epithelial cells and upregulate inflammatory responses, which can lead to ventilator-induced lung injury.^{4,5} In asthmatic airways, compressive mechanical forces resulting from smooth muscle constriction have been shown to cause airway epithelial cells to communicate with neighboring mesenchymal cells to initiate and amplify airway remodeling.^{6,7}

Over the past decades, discovery and elucidation of these mechanosensitive events in various physiologic and pathologic situations have been of paramount importance and interest in respiratory biology and physiology. Experimentally investigating the effects of mechanical forces on pulmonary epithelial cells, however, is often challenging for several reasons: (i) Due to the complex architecture and mechanical properties of the lung and marked spatial heterogeneity of pulmonary epithelial cells, *ex vivo* and *in vivo* models based on whole organ systems are difficult to use for studying the mechanical responses of airway and alveolar tissue at the cellular level. Instead, *in vitro* cell culture systems can be used as an alternative approach. Unlike other traditional culture methods, however, *in vitro* culture of pulmonary epithelial cells often requires permeable supports for cell attachment and independent access to both basal and apical sides of a cellular monolayer to allow the cells to polarize. Moreover, after reaching confluence, the cells need to be grown at an air-liquid interface to differentiate and gain morphological and biochemical phenotypes that match those found in native tissue. (ii) It is difficult to precisely reproduce *in vivo*-like dynamic mechanical microenvironments of pulmonary epithelial cells *in vitro*. This usually entails careful design, fabrication, and engineering of mechanical tools capable of generating different types of physical forces that can modulate the interactions of the epithelial cells with culture substrates or fluid flow to simulate various physiologic and pathologic conditions. Also, the need for the ability to systematically control the level of applied forces and to quantify actual mechanical stresses acting on the cells remains an important issue in such systems. (iii) Mechanical forces in the respiratory system can induce not only immediately-detectable changes in the morphology and viability of pulmonary epithelial cells, but also very transient signaling events mediated by soluble molecules such as cytokines and growth factors. This can trigger complex intra- and intercellular signal transduction leading to longer-lasting effects on the cells. Therefore, a detailed understanding of the cellular responses to mechanical stimulation requires accurate and quantitative measurements and analysis of the biochemical environments of pulmonary epithelial cells. Such tasks usually call for the development of biological assay systems with high sensitivity and specificity.

These challenges and requirements have limited our understanding of the behavior of lung cells in their mechanically active microenvironment and in turn, driven the development of new methodologies to address the technical limitations arising from the lack of appropriate tools for cellular studies. In this concise review, we describe recent research efforts directed towards

developing new experimental models that enable *in vitro* engineering of pulmonary tissues, recreation of their dynamic mechanical environment, and measurements of their response to various physiologic and pathologic mechanical forces. In particular, our discussion will focus on recent advances in the development of microengineered lung cell culture models known as lung-on-a-chip designed to mimic structural, environmental, and functional complexity of living human lungs. We will first present early studies that demonstrated the proof-of-principle for creating microengineered biomimetic models of the lung. This will be followed by the examination of representative examples of lung-on-a-chip models drawn from the recent literature. Finally, we examine future prospects and barriers to progress in biopharmaceutical applications of lung-on-a-chip technology.

13.2 Early Demonstration of Lung-on-a-chip: Airway Crackle-on-a-chip

Formation of physiological lung tissue *in vitro* is often challenging due to the need to culture lung epithelial cells at the air–liquid interface (ALI) required for cell differentiation. While tissue culture inserts containing permeable membranes supports (*e.g.*, Transwell inserts) provide a robust platform for ALI culture of lung cells, most of these systems fail to recapitulate the dynamic environment of the respiratory system. Early lung-on-a-chip studies focused on demonstrating the possibility of using multilayered microfluidic devices to address this important limitation. A human small airway-on-a-chip model developed by the Takayama group provides a representative example of such devices.⁸ To enable experimental investigation of airway closure-induced lung injury, the team designed a compartmentalized microfluidic device in which a porous membrane was sandwiched between two PDMS microchannels (Figure 13.1A). This device architecture permitted long-term (>3 weeks) ALI culture of primary human small airway epithelial cells (SAECs) to produce a differentiated airway epithelium with structural integrity and barrier function. More importantly, this culture system was integrated with a microfluidic plug generator capable of creating microscopic liquid plugs (Figure 13.1B) that were actuated to propagate over the epithelial surface and rupture to mimic closure and reopening of small airways due to mucus plugs in diseased lungs. This dynamic airway-on-a-chip model showed that plug propagation and rupture can generate abnormally large mechanical forces and harm the epithelium in a dose-dependent manner (Figure 13.1C). Another important finding was that plug rupture generated pressure waves similar to those of abnormal breathing sounds known as respiratory crackles. Based on these results, it was suggested that respiratory crackles may be associated with mechanical lung injury due to frequent airway closure and reopening. Combined with the engineering novelty of the system, this new physiological insight provided strong evidence for the advantage and potential of lung-on-a-chip systems as a novel research platform.

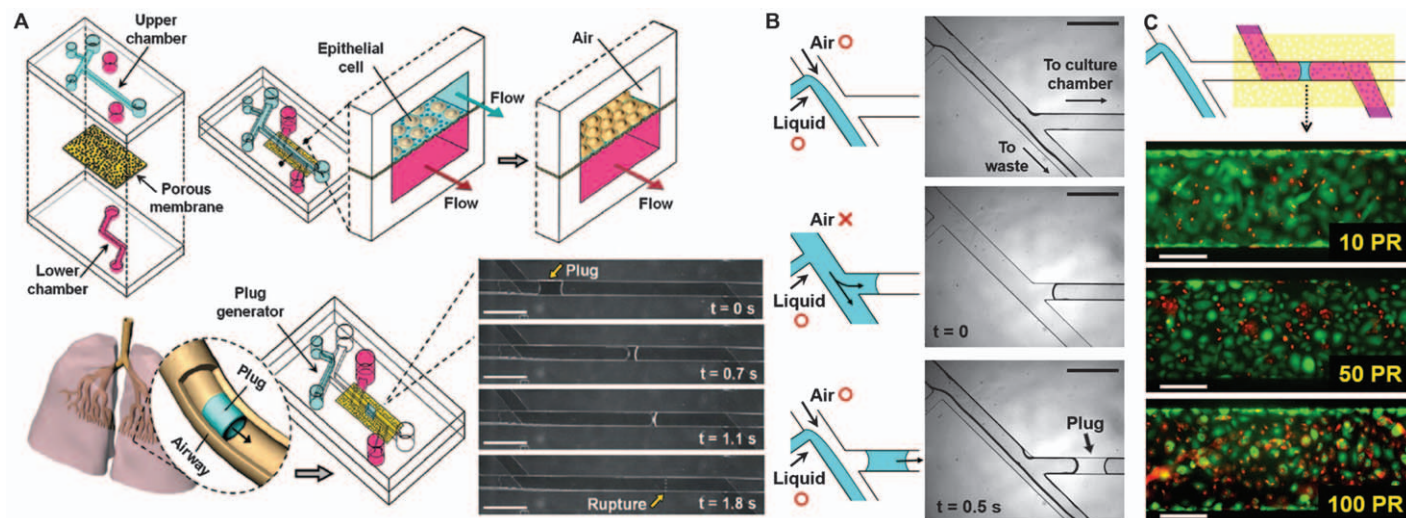


Figure 13.1 A. A human small airway-on-a-chip designed to simulate liquid plug propagation and rupture during airway reopening. B. Generation of microscopic liquid plugs in the microfluidic plug generator using high-speed air-liquid two-phase flow. Scale bars, 1 mm. C. Injury responses of the airway epithelial cells to plug propagation and rupture. Green and red show live and dead cells, respectively. PR represents the number of plug propagation and rupture events over 10 minutes. Scale bars, 150 μm .⁸

13.3 Human Breathing Lung-on-a-chip

This early demonstration was followed by another major study that showed for the first time the feasibility of reconstituting complex and integrated organ-level physiological responses of the lung in a microengineered device (Figure 13.2A and B).⁹ This system was designed to mimic the mechanically active alveolar-capillary unit of the living human lung. The device was created in a multi-compartment microfluidic system in which human alveolar epithelial cells were cultured in close apposition to primary human pulmonary microvascular endothelial cells on a thin porous elastomeric membrane to form a barrier tissue reminiscent of the alveolar-capillary interface *in vivo*. Inspired by the mechanism of breathing in the lung, the authors also devised a novel mechanical actuation scheme based on controlled application of vacuum to cyclically stretch the microengineered alveolar-capillary barrier and to mimic physiological breathing motions. Importantly, this study demonstrated the novel capability of the human breathing lung-on-a-chip model to mimic complex integrated organ-level responses. For example, when the alveolar epithelium was exposed to pathogenic bacteria in this microdevice, the epithelial cells released inflammatory cytokines and activated the microvascular endothelial cells on the opposite of the membrane, inducing them to express high levels of adhesion molecules such as ICAM-1. Primary human neutrophils circulating in the lower capillary channel recognized these activated endothelial cells and established firm adhesions to the endothelium, after which the adhered neutrophils underwent transmigration across the alveolar-capillary barrier (Figure 13.2C). The transmigrated neutrophils then appeared in the upper alveolar compartment and phagocytosed the bacterial cells on the epithelial surface. In addition, the ability of this model to recapitulate dynamic mechanical activity of the lung led to the discovery of previously unexplained adverse effects of physiological breathing-induced mechanical forces on inflammatory and injury responses. For example, in nanotoxicology studies using silica nanoparticles that simulated air pollutants, cyclic breathing motions in the lung-on-a-chip system substantially increased endothelial expression of pro-inflammatory adhesion molecules and intracellular production of reactive oxygen species, suggesting the promotive effects of physiological breathing on acute toxic responses to environmental particulates. This biomimetic microdevice also revealed considerable increases in the translocation of nanoparticles from the alveolar airspace to the vascular compartment due to breathing-associated mechanical strain.

This advanced lung-on-a-chip platform was used in the follow-on study to test the feasibility of engineering specialized models of lung diseases. To this end, the authors created a microengineered model that simulated the development and progression of pulmonary edema induced by dose-limiting toxicity of a chemotherapeutic drug, interleukin-2 (IL-2).¹⁰ When the vascular microchannel lined with human microvascular endothelial cells was treated with clinical doses of IL-2, this microdevice replicated the leakage of

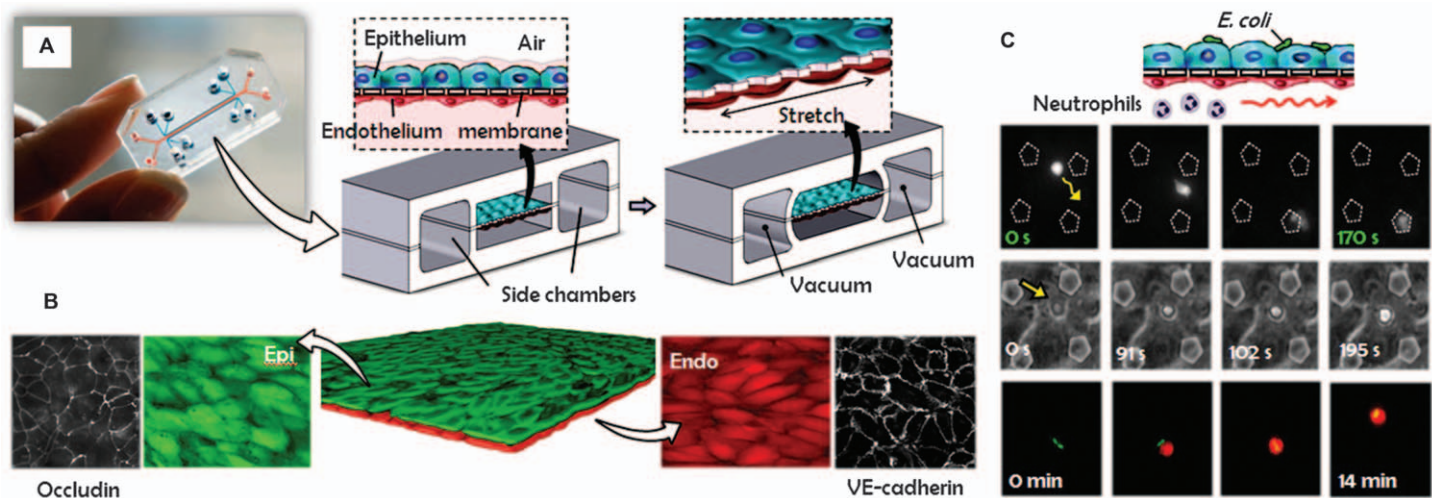


Figure 13.2 A human breathing lung-on-a-chip. A. The microfabricated lung mimic device recreates physiological breathing movements by applying a vacuum to the side chambers and causing mechanical stretching of the PDMS membrane forming the alveolar-capillary barrier. B. Long-term microfluidic co-culture produces a tissue-tissue interface consisting of a single layer of the alveolar epithelium (Epi; green) closely apposed to a monolayer of the microvascular endothelium (Endo; red), both of which express intercellular junctional structures such as occludin or VE-cadherin. C. Neutrophils flowing in the lower vascular channel adhere to the endothelium activated by *E. coli* in the alveolar chamber, transmigrate (top row), emigrate into the alveolar space (middle row), and engulf the bacteria (bottom row).⁹ Reproduced from ref. 9 with permission from the American Association for the Advancement of Science, Copyright 2010.

intravascular fluid into the air-filled alveolar compartment and concomitant flooding of the air space. Intra-alveolar deposition of fibrin clots due to activation of coagulation cascades was also observed in this model, which is another clinically-relevant toxic response during the course of IL-2-induced pulmonary edema. High-resolution microfluorimetric analysis of the alveolar–capillary interface showed that the IL-2 toxicity-induced edematous responses are elicited by compromised intercellular junctions and resultant increases in barrier permeability. Furthermore, this mechanically active model capable of mimicking physiological breathing motions revealed the promotive effects of breathing-generated mechanical forces on IL-2-induced tissue injury, which was effectively inhibited by potential drug candidates such as angiopoietin-1 and a newly developed transient receptor potential vallinoid 4 (TRPV4) ion channel blocker.

13.4 Recent Advances in Lung-on-a-chip Technology

Early investigations described above exemplify an array of opportunities enabled by lung-on-a-chip technology for prediction and mechanistic investigation of complex physiological responses of the human lung. Building upon the success of these studies, researchers have recently reported several lung-on-a-chip systems that model various aspects of lung physiology and pathophysiology. Here we describe representative examples of these models.

13.4.1 A Microfabricated Organotypic Lung Model for the Study of Host–Pathogen Interactions

Lung-on-a-chip technology provides new opportunities to model pathophysiologically-relevant host-pathogen interactions by allowing for co-culture of lung cells with living bacteria, virus, or fungi in a controlled manner. A recent example of modeling host–pathogen interactions in the lungs can be found in the work of Barkal *et al.* that established a micro-engineered model of the human bronchiole used to study the early stages of *Aspergillus Fumigatus* infection in human lungs (Figure 13.3A).¹¹

This device consisted of a single compartment through which three PDMS rods were inserted to form patent lumens upon filling the compartment with collagen hydrogel and subsequent removal of the rods. A central lumen was seeded with SAECs to form the bronchiole, and microvascular endothelial cells were added to the two remaining lumens to form perfusable vessels. Additionally, fibroblasts were embedded in the surrounding collagen hydrogel to model the stromal tissue compartment. While the outputs of their study do not emphasize fibroblast responses to infection, it is likely that the organotypic mixture of epithelial, interstitial, and stromal cell types creates a more physiologically-relevant milieu for the study of host response to infection. To model the small airway response to inhaled pathogens, the central airway lumen was exposed to *Aspergillus Fumigatus*, a common fungal pathogen responsible for respiratory infections. Following exposure, the

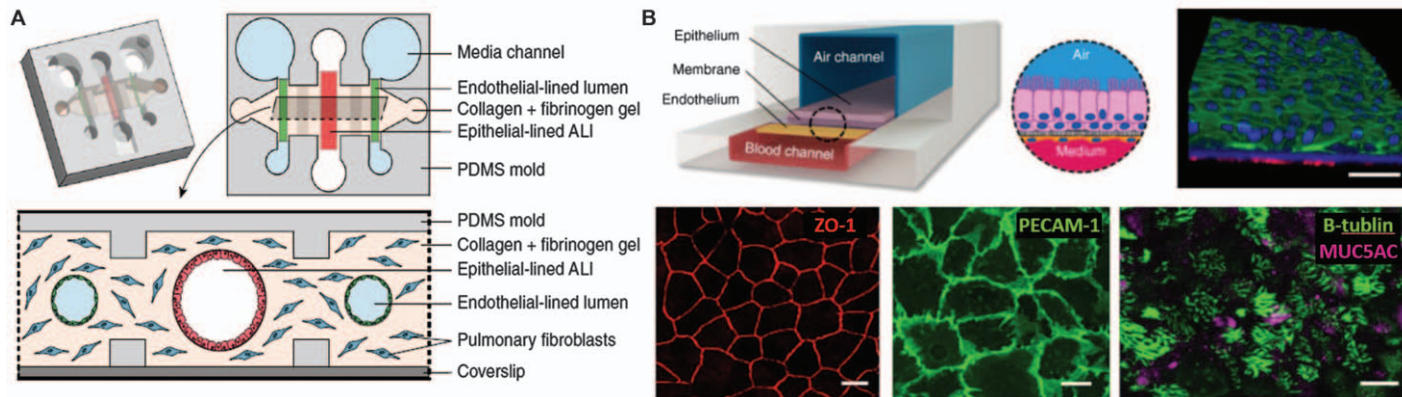


Figure 13.3 A. An *in vitro* model of human airway containing an organotypic mixture of airway epithelial cells, lung fibroblasts, and endothelial cells in a physiological 3D environment.¹¹ B. A microengineered model of human small airway composed of fully differentiated human SAEs that for tight barrier tissue and exhibit mucociliary function.¹² Part A reproduced from ref. 11, <https://doi.org/10.1038/s41467-017-01985-4>, under the terms of the CC BY 4.0 licence, <https://creativecommons.org/licenses/by/4.0/>. Part B reproduced from ref. 12 with permission from Springer Nature, Copyright 2015.

fungus traversed the epithelial barrier and formed typical fungal hyphae structures in the surrounding collagen hydrogel. To model the first line of host defense, human neutrophils were added to the perfused microvascular lumens where some cells were shown to enter the surrounding hydrogel and migrate towards fungal hyphae structures.

To drive continued advancement in the field, organs-on-chips will need to facilitate the study of increasingly complex aspects of pathogenesis. An example of this is microbial volatile communication which entails crosstalk between multiple pathogens, often across kingdoms, and occurs in the context of pulmonary infection when fungal pathogens are introduced into the lung of an individual with chronic low-grade bacterial infection. Leveraging the inherent capacity of microengineered models to physically separate and fluidically connect different types of cells or microbes, the authors created a closed device containing separate chambers of *Aspergillus Fumigatus* and the bacterium *Pseudomonas Aeruginosa* along with three human bronchiole modules to facilitate volatile communication between the pathogens and the airway epithelial cells *via* open airway lumens. Interestingly, this study demonstrated increased epithelial cytokine production in response to microbial volatiles without direct pathogen exposure. While this work successfully recapitulated human pulmonary fungal infection *in vitro*, much additional progress is needed to realize clinically-relevant models of pulmonary infection that will help inform a better understanding of how to boost the early host response to pathogen exposure.

13.4.2 A Microengineered Model of Human Small Airways

Lung-on-a-chip model systems enable unprecedented study of the synergistic crosstalk between multiple cell types that drive pathological processes in the human lung as demonstrated by the work of Benam *et al.* (Figure 13.3B).¹² In this study, the research team engineered a human ‘small airway-on-a-chip’ comprised of two adjacent microfluidic channels separated by a porous membrane to study the influence of epithelial–endothelial crosstalk on inflammatory cytokine production and neutrophil recruitment in the small airways of patients with diseases such as asthma and chronic obstructive pulmonary disease (COPD). The upper microfluidic channel of this device was seeded with human SAECs later cultured at the air–liquid interface to facilitate mucociliary differentiation, while the lower microfluidic channel was seeded with lung microvascular endothelial cells and perfused to create a physiological milieu capable of modeling organ-level inflammatory disease processes. First, to model goblet cell hyperplasia, cytokine hypersecretion and mucociliary dysfunction seen in the small airways of asthmatics, supraphysiological concentrations of IL-13 were added to the small airway-on-a-chip *via* the medium perfused in the microvascular channel. Interestingly, IL-13 stimulated cytokine release by airway epithelium or microvascular endothelium cultured alone in

monoculture devices, while the full device assembly with both tissue layers revealed synergistic pro-inflammatory crosstalk that resulted in total cytokine levels significantly increased above the sum of levels measured in monoculture devices. The use of COPD patient-derived cells in this device recapitulated key disease features including selective cytokine secretion, increased neutrophil recruitment, and exacerbation-like increases in these responses upon introduction of viral and bacterial pathogens. This system was also treated with IL-13 to create a disease model that recapitulated certain features of asthma, which was used to demonstrate quantitative measurement of clinically-relevant outputs upon treatment with anti-inflammatory compounds, including reduced cytokine production, decreased goblet cell hyperplasia, and recovery of normal ciliary beat frequency. Furthermore, culture of COPD airway cells in this platform enabled simulation and analysis of neutrophil recruitment to the microvascular endothelium driven by epithelial cytokine secretion, demonstrating the capacity to measure and modulate ‘druggable’ aspects of clinical exacerbations driven by neutrophilic inflammation in the lungs of COPD patients.

13.4.3 A Specialized Disease Model of Lung Cancer

In vitro microphysiological systems offer the promise to serve as alternatives to animals for the study of cancer. Animal studies often suffer from their limited ability to precisely control, vary, and elucidate the contribution of microenvironmental cues to tumor progression. The work of Hassel *et al.* describes an adaptation of the previously developed breathing lung-on-a-chip system containing two microfluidic channels separated by a cyclic stretched porous membrane to create a human orthotopic model of non-small-cell lung cancer (NSCLC) that recapitulates cancer behaviors specific to the organ microenvironment (Figure 13.4A).¹³ Interestingly, the group reported that growth of lung cancer cells was significantly suppressed (by more than 50% in comparison to static culture devices) when the cells were mechanically stretched. This study raises the possibility of a positive feedback loop during the progression of lung cancer in which the loss of motion in the lung due to NSCLC cell proliferation into alveolar spaces causes accelerated tumor growth. In addition to physiologically-relevant cytokine secretion profiles, mechanical actuation of the devices to mimic breathing motions was also shown to recapitulate the resistance of NSCLC tumor cells with an EGFR T790M point mutation to irreversible tyrosine kinase inhibitor (TKI) therapy in patients with late-stage disease. This was in contrast to a traditional static mode in which NSCLC cells were seemingly responsive to the administered TKIs even at low drug doses. The ability to faithfully recapitulate *in vivo* pathophysiological phenotype of NSCLC tumors and their responses to drugs demonstrates the potential of this model as a predictive screening platform for the development of more effective anticancer therapeutics.

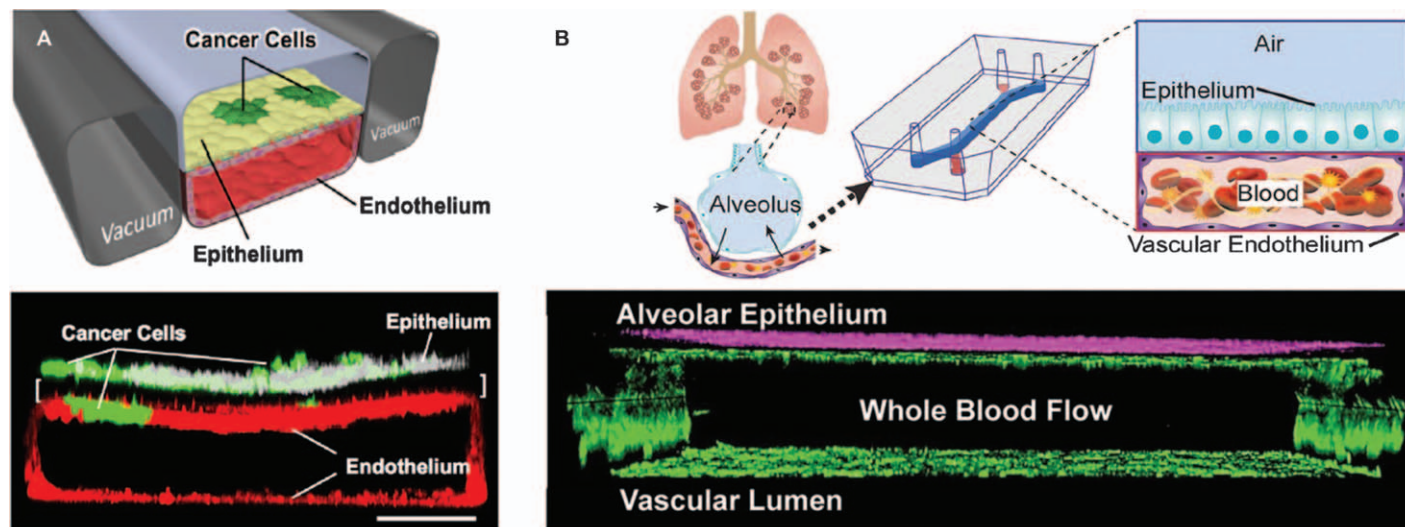


Figure 13.4 A. A microphysiological model of lung cancer composed of lung cancer cells cultured with normal alveolar epithelial cells and pulmonary vascular endothelial cells in a multilayered microfluidic cell culture chamber.¹³ B. An alveolus-on-a-chip for the study of thrombosis.¹⁴

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13.4.4 A Microfluidic Model of Intravascular Thrombosis in the Alveolar System

Although pulmonary thrombosis is a significant cause of patient mortality, there exist no traditional *in vitro* models of microvascular thrombi formation. Additionally, animal models of pulmonary microvascular thrombosis fail to capture the complex hemodynamic behavior of human lungs. To address these limitations, Jain *et al.* presented a microfluidic alveolus-on-a-chip model that recapitulated the organ-level pathophysiological effects of pulmonary thrombosis (Figure 13.4B).¹⁴

In this microsystem, the alveolar–capillary interface was reproduced with two rectangular PDMS compartments separated by a thin, extracellular matrix-coated porous membrane. Primary human alveolar epithelial cells were cultured at the air–liquid interface along the membrane in the upper compartment, and human umbilical vascular endothelial cells (HUVECs) lined all four sides of the lower compartment to form a continuous lumen. This platform enabled the perfusion of whole blood through the lower vascular compartment without thrombus formation nor platelet adhesion in healthy engineered microvessels. Following stimulation with tumor necrosis factor- α (TNF- α), however, the system exhibited thrombus formation and rapid platelet recruitment. The aggregating platelets in this condition also formed a tear-drop shape that mirrored *in vivo* observations, which is absent in ECM-coated microfluidic devices traditionally used to study thrombus formation. Additionally, the system was used to study lipopolysaccharide (LPS) endotoxin-induced thrombus formation, revealing that tissue–tissue interactions between the alveolar epithelium and vascular endothelium mediate the prothrombotic response through an epithelium-generated cytokine cascade.

Lastly, the application of the alveolus model in preclinical drug development was shown by demonstrating the cytoprotective and antithrombotic activity of permodulin-2 (PM-2), a potent inhibitor of inflammation-mediating protease activated receptor-1 (PAR-1), in the presence of LPS.

13.5 Future Opportunities and Challenges

The lung-on-a-chip models outlined in this review highlight the feasibility of developing *in vitro* model systems to recapitulate the complexity of the respiratory system in ways that have not been possible using traditional *in vitro* techniques. As is evident from the studies described here, understanding various physiological responses to the dynamic environment of the lung has been greatly facilitated by the lung-on-a-chip systems that can reproduce, manipulate, and measure the interaction of living lung tissues with physiologically-relevant mechanical and biochemical cues.

From the stand point of lung biology and physiology, the lung-on-a-chip technology is well-poised to provide a novel research platform for investigating the influence of the dynamic environment of the lung, especially mechanical forces, in various physiological and disease contexts. Advancing

our knowledge of the mechanically regulated behavior of the lung will inevitably require careful design and engineering of new systems that make it possible to better mimic the geometrical and structural properties of pulmonary airway and alveolar tissues and to precisely recreate the essential feature of tissue deformation and fluid flows occurring *in vivo*. Assay systems capable of detecting mechanotransduction signaling molecules with high sensitivity and specificity will also make a significant contribution to such efforts by providing a means to study the molecular mechanisms of observed mechanosensitive events. The development of these new tools will likely involve contributions from various scientific and engineering fields.

From a broader perspective, the lung-on-a-chip may serve as a technical basis for developing predictive drug screening platforms. Recent years have witnessed increasing recognition of organ-on-a-chip technology as a driver of innovation in the pharmaceutical industry that is greatly burdened with ever-increasing costs of research and development. This major problem stems mainly from high attrition rates at the later and more costly stages of clinical drug development. One of the promising approaches to addressing this issue is to improve the predictability and reliability of preclinical drug testing in order to identify compounds that are destined to fail in clinical trials. Animal studies commonly required for efficacy and safety testing are costly, time-consuming, and require large amounts of compounds that are often not available at the earlier stages of drug development. More importantly, traditional animal testing often fails to predict human drug responses, and many now question the ethics of sacrificing animals without reliable predictions for clinical outcomes. For example, cardiac toxicity by non-cardiac drugs is the most common cause of drug development delays, FDA failure, and market withdrawal, yet it is difficult to immediately detect adverse cardiac effects in animals or using traditional *in vitro* models. In fact, the lack of success with existing drug screening and safety assays has pushed major pharmaceutical companies to reassess cardiovascular drug discovery as both intractable and unprofitable. They have determined that it is difficult to identify effective drugs with the current set of product development and testing tools. As a result, they have essentially chosen to abandon the field. Although other companies and research laboratories worldwide continue to develop and test new medical products, frustration with their existing dependence on costly and time-consuming animal studies that often do not effectively predict clinical outcome is a common experience.

Unfortunately, the poor predictive power of these preclinical animal models often leads to the failure of drug compounds late in their development, only after they enter human clinical trials. As a result, the current cost of developing a new drug is estimated to be \$800 million, over 60% of which is caused by preclinical testing using traditional cell culture and animal studies. The use of live animals also has been a mainstay of toxicology focused on studying the potential adverse effects of chemicals, food additives, pesticides, cosmetics, and other substances. Animal toxicology also suffers from high costs (*e.g.*, ~\$2–4 million per carcinogen test), time delays (*e.g.*, ~4–5 years for

carcinogen testing), outdated guidelines, poor reliability, and weak relevance to human physiology, which have led to significant public opposition. To overcome these drawbacks, toxicologists have begun to seek alternatives to animal testing, as exemplified by the report of the US National Academy of Sciences in 2007 calling for a major paradigm shift in toxicology that would rely less heavily on animal studies and instead focus on new *in vitro* methods. Organ-on-a-chip technology reviewed here is well-poised to provide more predictive human relevant models for toxicity testing and to contribute to gaining insights into the mechanisms of toxicity at organ levels.

In vitro models have already enabled prediction of absorption, distribution, metabolism, and excretion (ADME) of many drug candidates. The successful implementation of these tools substantially reduced failure rates in the clinic due to poor pharmacokinetic properties or bioavailability. However, failures due to lack of efficacy and toxicity have not improved. Organ-on-chip microsystems, including lung-on-a-chip models, hold great potential for the development of human relevant disease models for screening drug efficacy. These microengineered disease models could profoundly influence various stages of drug development, including target selection and target validation, lead identification, and candidate selection. In addition, the systems could also provide integrated organ systems to study interplay of different organ in determining ADME properties of compounds. Currently this is not possible with conventional *in vitro* systems.

Implementation of lung-on-a-chip technology as a new paradigm for drug screening will require extensive validation and perhaps decades of data generation to build confidence and to receive approval from the regulatory authorities. Tackling this challenging process will be greatly facilitated by collaboration and partnership between academic investigators, pharmaceutical companies, and regulatory agencies. On a technical level, advancement of lung-on-a-chip technology will entail significant advances in systems engineering and bioinstrumentation to (i) automate the operation of microfluidic cell culture, (ii) increase the throughput of culture and bioassays, and (iii) improve spatiotemporal resolution of sensing and monitoring of biological responses. In addition, sourcing of human lung cells is another key challenge that may be addressed by emerging alternatives to primary human cells such as inducible pluripotent stem cells.

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CHAPTER 14

3D Tissue Model of Cancers

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14.1 Introduction

3D *in vitro* models have been widely used in cancer research to provide a bridge between 2D platforms such as petri dishes and delicately processed xenografts (animal models) to reconstitute human cancer tissue in the cancer microenvironment. Among the many reasons why 3D *in vitro* models are so valued compared to previous 2D models, the foremost should be the capability of 3D models to recapitulate actual human cancer microenvironments in a deconstructive manner. Such 3D *in vitro* models offer an alternative approach to both 3D and 2D culture methods: for 3D, studying complex whole tissue organs in depth, and for 2D, overcoming limitations in space control, cell-to-cell interaction, cell adhesion and confounded cell morphology. Ways to acquire more physiological *in vivo* 3D cancer models are: to co-culture cancer cells under cancer microenvironments with stromal cells¹ and extracellular matrix components;² to precisely regulate oxygen conditions (rich, free or gradient) to mimic real oxygen levels found in native cancer tissues;³ and to culture immune cells to alter behaviors of cancer cells regarding proliferation, invasion and EMT (epithelial mesenchymal transition) and morphogenesis of stromal cells including angiogenesis and migration. In this chapter, limitations of 2D cultures compared to *in vivo*

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models will be discussed, as well as methods and strategies to build 3D *in vitro* models with specific references to show recent advancements.

14.2 About Cancer and the Microenvironments Shaping the Cancer

Cancer is the abnormal proliferation of genetically mutated cells. Cancer can be categorized into hundreds of different kinds based on the locations where the cancer is located. As the cancer in certain location starts to affect physiological functions in our body, these specific physiological functions begin to degenerate.⁴ The reason why it is so hard to fight against the cancer is that most of the time, cancer is detected after the effects of cancer progression are observed, except in cases of early detection. The most important key factor for cancer is whether it is a benign tumor or a malignant tumor (Figure 14.1). A benign tumor, not much different from the neighboring cells, confines itself to the original spot, neither invading surrounding normal tissue nor spreading to different sites in the body. Thus, benign tumors do not cause major harm to the body as they can be easily removed.⁵ On the other hand, a malignant tumor has the capability to invade surrounding tissue and spread to different parts of the body, putting the patient's life in danger.⁶ The foremost characteristics of a malignant tumor are that it can spread into different sites through the blood stream and lymphatic systems (metastasis) (Figure 14.2).^{7,8} The term 'cancer' refers to such malignant tumors. Although benign tumors are usually removed through surgical means, malignant tumors are very much resistant to

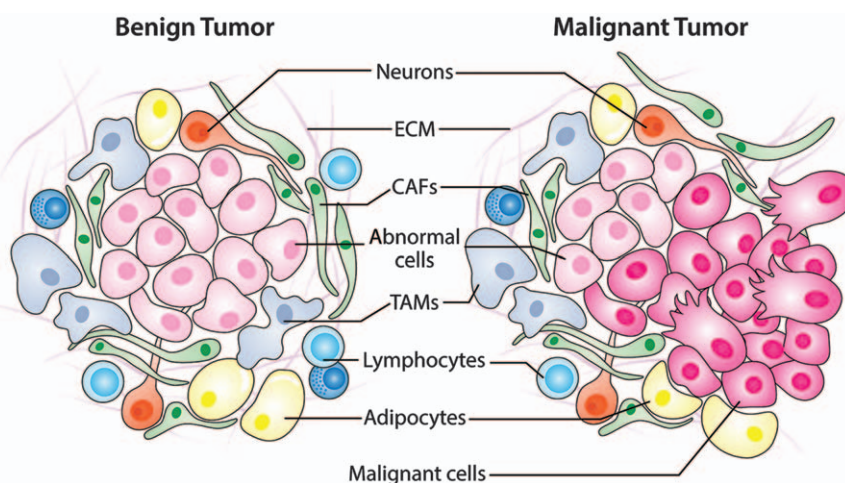


Figure 14.1 The cellular components of benign tumors and malignant tumors. Locally grown benign tumor cells cannot spread throughout body whereas, malignant tumor cells invade and metastasize to different parts of our body.

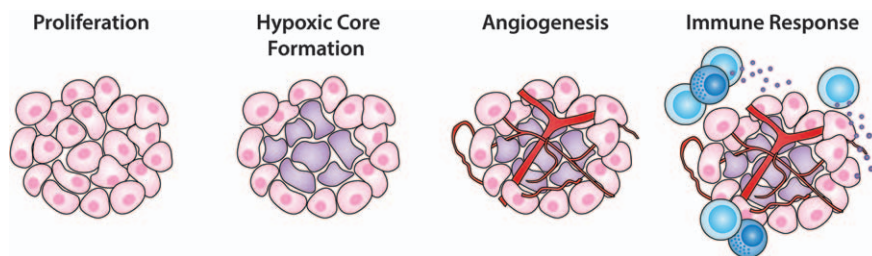


Figure 14.2 The progression of tumor metastasis. First, cancerous cells appear and grow. Evading anti-tumor immune responses, these cancerous cells survive and even thrive. In order to acquire nutrients and oxygen, cancer cells cause defective blood vasculatures. After the formulation of blood vessels, some cancer cells invade into blood vessels causing further inflammation and metastasis.

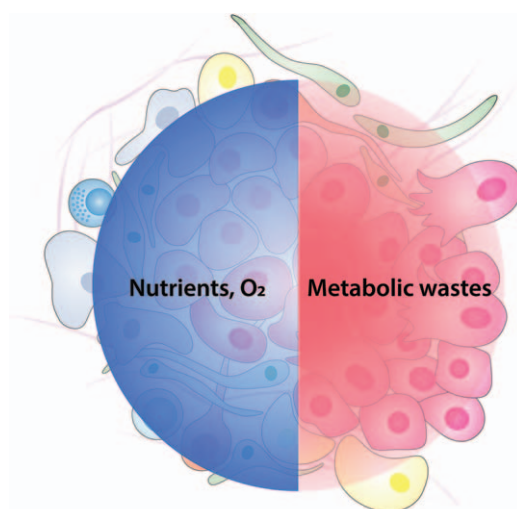


Figure 14.3 Schematics representing various gradients in the tumor microenvironment. When compared to normal tissue, tumor tissue can be characterized with low oxygen tension, higher acidity, defective vasculature, and different composition of ECM.

various treatments currently available; 3D modeling may lead to new, successful treatment methods.

As all human tissues and organs are three-dimensionally formed, having 3D models of cancer available is pivotal. In order to form 3D models of cancer, one must understand the microenvironments that actually shape cancer (Figure 14.3). The cancer microenvironment is mainly composed of different kinds of stromal cells and the remodeled extracellular matrix (ECM).^{9,10} The major stromal cells (connective tissue cells in organs) that play important roles in cancer progression are cancer-associated fibroblasts, various immune cells, pericytes, endothelial cells and adipocytes. All these

stromal cells offer growth factors to the cancer to allow it to proliferate with diversity, leading to drug resistance and disease progression. An increased relativity between stroma and cancer has associated stronger tumor progression followed by decreased patient survival rates.^{11–14} Therefore, to mimic cancers in 3D models, acquiring tumor heterogeneity and phenotypic characteristics of original cancer sites are crucial.

14.2.1 Cancer-associated Fibroblasts

Let us say a certain tissue in an organ is scarred. Then, these scarred tissues undergo a wound-healing process by differentiating neighboring fibroblasts into myofibroblasts, possibly inducing organ fibrosis.^{15,16} When organ fibrosis chronically progresses, there is a high risk of retaining cancer developments.¹⁷ As such, myofibroblasts are predominantly present in a tumor microenvironment. These myofibroblasts and fibroblasts abundantly found in a cancer are called cancer-associated fibroblasts (CAFs). CAFs are primarily derived from residential fibroblasts (Figure 14.4), however CAFs also come from various sources such as endothelial cells, pericytes, smooth muscle cells, mesenchymal stem cells or even its own cancer cells that have been gone through EMT.^{18,19} Among the heterogeneous functions and phenotypes of CAFs, primary roles are to produce a number of growth factors to aid tumorigenesis and to remodel the ECM around tumors. Growth factors such as transforming growth factor B (TGF- β), fibroblast growth factor (FGF), hepatocyte growth factor (HGF) *etc.* are all secreted once the CAFs are

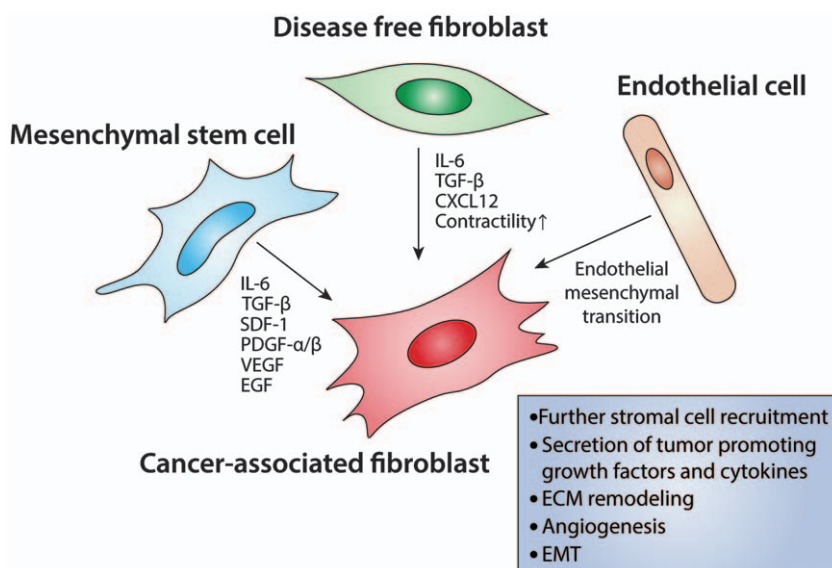


Figure 14.4 A schematic showing the origins of CAFs. Neighbor fibroblasts are the main sources of CAFs, however, they are also generated from other cell types.

up and running.^{19,20} All these growth factors are secreted for one reason: to promote tumorigenesis; TGF- β for inducing EMT such that immune cells cannot affect the cancer cells, FGF and HGF for acting as mitogen agents in tumors. Besides its ability to secrete growth factors to promote cancers, CAFs produce substantial ECM components and enzymes that actually remodel the surrounding ECM.²¹ For example, one of the secretions from CAFs is Collagen type 1 which plays a critical role in decreasing chemotherapeutic drug uptakes and regulating tumor sensitivity.²² As it is now known that CAFs are an indispensable factor in tumors, regulating CAFs can actually induce tumor necrosis, possibly increasing survival rates of patients. Recent research has shown that targeting fibroblast activation protein (FAP) through the use of a DNA vaccine can tremendously suppress tumor outgrowth in murine breast carcinoma.²³ Furthermore, inhibiting NF- κ B, activated by pro-inflammatory factors, can withdraw tumor progression.²⁴

14.2.2 Immune Cells

The immune system is perhaps the most important barrier that a tumor has to go through while its adapting. The most widely-accepted notion regarding the relationship between immune systems and tumors is ‘immunoediting’.²⁵ Immunoediting is the process of tumors surpassing innate immune systems of its host. There are three major stages in immunoediting: elimination, equilibrium, and escape (Figure 14.5).²⁶ During the first stage of elimination, all kinds of immune cells including lymphocytes, macrophages, natural killer cells, and dendritic cells are gathered to the tumor site. While all immune cells are gathered at the origins of tumor, lymphocytes endogenously produce interferon- γ (IFN- γ) which fights against the formation of spontaneous tumors. The role of IFN- γ is very important as IFN- γ promotes tumor recognition and the eradication of tumors.²⁷ For example, in the paper by Dighe *et al.* the growth of immunogenic sarcomas transplanted in mice proliferated at much faster rate than the control mice once the neutralizing monoclonal antibody to IFN- γ was injected.²⁸ As such, macrophages which engulf the tumor and start the specific defense mechanisms, natural killer cells (or natural killer T cells) which directly kill the tumor, dendritic cells which interact with T cells and B cells to begin immune responses and all kinds of immune cells are activated in the elimination process.²⁷ As the equilibrium phase initiates, tumors acquire abilities to resist immune detection or elimination even though host immune systems are still in the process of elimination. This process of equilibrium is actually the longest process of the three stages. Lymphocytes and IFN- γ are key factors in immune selection as many variate tumors cells are killed. However, new ones with resistive mutations are spawned as well.²⁹ As a consequence, the tumors start to expand with less hostility. Further into the equilibrium, the tumor now actually escapes the surveillance of the immune system with genetic and epigenetic transformations.³⁰ Besides immunoediting, one of the most breakthrough methods in cancer therapy is immunotherapy using immune checkpoint. Since

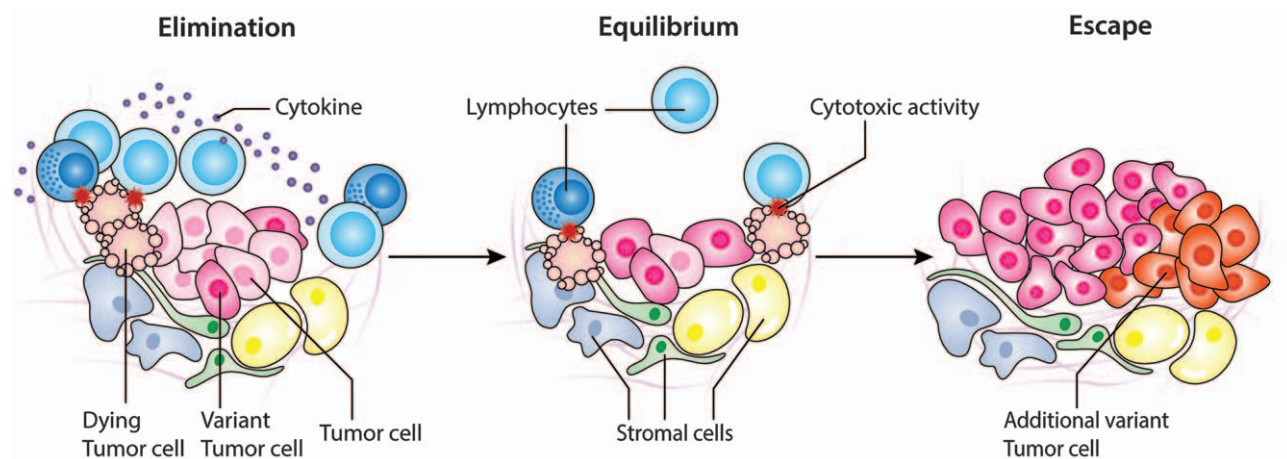


Figure 14.5 Three stages of 'cancer immunoediting' theory. In the first stage, elimination, host immune cells produce strong anti-tumor immune responses, leading to successful elimination of neoplastic cells. However, in some cases, these various neoplastic cells survive elimination due to immune modulatory functions causing immuno-evasion. Further elimination is undergone in the equilibrium phase. At the end, cancerous cells vulnerable to the anti-tumor immune response have all been removed and only surviving cancerous cells reach the escape phase. In the escape phase, cancer cells now avoid any anti-tumor immune responses.

numerous immune cells are turned 'off' in tumors, by trimming the 'off' switch through gene editing within immune cells in tumors, these immune cells regain the ability to attack previously ignored tumor cells. In a published paper by Menger *et al.*, mice with genetically edited immune cells survived more than 70 days whereas the control mice all died.

14.2.3 Vascular Endothelial Cells

Originally, the blood vessels serve to deliver and circulate oxygen, nutrients, waste, and even immune cells. However, with tumors, blood vessels function somewhat differently.³¹ As with any tissue in the body, tumors also require vessels through angiogenesis and vasculogenesis for the transportation of oxygen, nutrients, waste *etc.* However, all aspects of tumor vasculatures are very abnormal in structure and function.³² Heterogeneously vascularized, the vessel networks are sprouted in a chaotic manner such that lumen sizes are very uneven. On top of that, vessels are very 'leaky,' resulting in increased interstitial flow that creates random blood flows.^{31,32} Because of these characteristics, not only do drugs scarcely reach the tumor sites, but even nutrient and oxygen deliveries are reduced, causing necrotic and hypoxic environments. Another term that must be discussed regarding tumors is hypoxia.^{33,34} The reason that hypoxia is so important is due to its indispensable relationship with tumors: promotion of more angiogenesis in tumors, changes in metabolism, and increased resistivity to apoptosis and all sorts of cancer therapies.³⁵ In response to hypoxic conditions, hypoxia-inducible factors (HIFs) are produced which increase the metastasis ability in tumors. Furthermore, it has been shown that hypoxia causes changes in oxidative phosphorylation to glycolysis, which leads, in turn, to tumors producing lactate. This high level of lactate results in acidic environments for tumors, therefore, low pH conditions. Because of this acidic environment, residential normal tissues around tumors are destroyed, offering opportunities for tumors to metastasize.³³

14.2.4 Pericytes

Closely related to angiogenesis and vasculogenesis in the tumor micro-environment, pericytes (perivascular stromal cells, see Figure 14.6) are pivotal factors in supporting vessels by producing vascular endothelial growth factor (VEGF).³⁶ However, whether to eradicate or to promote pericytes within the tumor microenvironment must be controlled at a very careful level. When the number of pericytes becomes very scarce, vessels become 'leaky,' leading to an increase in interstitial pressure.³⁷ As discussed above, more leakiness leads to decreased tumor perfusion, in which oxygen and nutrients are infrequently delivered to the tumor site, therefore causing hypoxic conditions. Consequently, metastasis is induced further. Since the number of pericytes is closely correlated to the metastasis of tumor, pericytes should be further investigated.

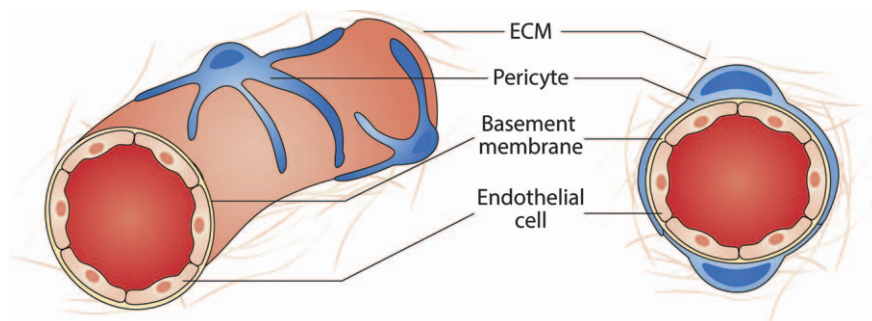


Figure 14.6 Schematics of pericytes. Pericytes reside just outside the endothelial cells and actively communicate with endothelial cells.

14.2.5 Adipocytes

Adipocytes are abundantly located in the breasts and abdomen and are one of the compactly-placed cells that form tumor microenvironments. Cancers like ovarian cancer have been reported to favor metastasis towards the omentum where adipocytes are abundant.³⁸ The driving factor behind such metastasis is adipokines called interleukin-8 (IL-8), tumor necrosis factor-alpha (TNF-), and interleukin 6 (IL-6).^{38,39} It has been confirmed that by coculturing adipocytes and ovarian cancer in either *in vivo* or *in vitro* conditions, the tumor growth was highly increased, implying that adipocytes actually play a role as energy sources. In more recent studies, when adipocytes were cocultured with breast cancer cells, free fatty acids from adipocytes which initiate fatty acid metabolism move to breast cancer cells, thereby increasing the cancer proliferation and migration.³⁸

14.2.6 Extracellular Matrix

Extracellular Matrix, also known as ECM, is critically important to tumorigenesis since ECM offers structural and biochemical supports to tumors. Although all tumors must form ECM as their niche, the composition of the ECM of each tumor is very much different from one another. Among the many different characteristics, commonly shared ones are: integrin binding, adhesion of a cell to the ECM, angiogenetic factors, cell-to-cell communication, 3D cell migration and invasion, and differentiation. For the purpose of cell culture, basement membrane extracts and collagen gels are often favored. One of the most widely-used basement membrane is Matrigel which comes from the mouse sarcoma tumor and has unique heterogenous compositions of structural proteins such as entactin, laminin, collagen type IV, and much more. In addition, reconstitute basement membrane shows differences between normal cancer cells and malignant cancer cells. In the case of breast cancers, these normal cancer cells differentiate and maintain their functions inside the basement membrane.⁴⁰ On the other hand, malignant breast cancers seem not to be affected by the basement membrane. More

interestingly, if a certain integrin, for example, B1 is removed from ECM, the malignant cancer cells revert themselves back into normal phenotypes.⁴¹

Besides reconstituted basement membrane, collagen type I is also widely used as an ECM that shows increased deposition during tumorigenesis. Acting as a blockade against cancer cell invasions, collagen type 1 is frequently used to study cell migrations and invasions since malignant cancer cells denature collagen to spread.⁴² Within collagen type 1, there are countless fibers forming collagen. These fibers, naturally placed randomly, become aligned formations when malignant cancer cells metastasize and this invasion rate is different based on the state of the collagen fibers.⁴³ As the collagen fibers become aligned, the stiffness of matrix increases, resulting in simultaneous increases in cell migrations through directional persistence and lesser protrusion of cancer cells.⁴⁴ Another reason for collagen stiffness is that because large numbers of CAFs are gathered around the tumor site, CAFs deposit more ECM components than any other close by tissues, increasing the stiffness of ECM.⁴⁵ Furthermore, of as much important as fiber alignments, the pore sizes of fibers decide the ability of cancer cells to invade to adjacent locations. When the pore sizes of collagen fibers are large, the cancer invasion is more encouraged with increased matrix stiffness. On the other hand, when the pore sizes of collagen fibers are small, the cancer invasion is disturbed with increased matrix stiffness.⁴⁶ While cancer cells continue their invasions, they produce various proteases such as matrix metalloproteases (MMP), serine proteases, and threonin proteases to destruct and remodel ECM. One of the proteases, MMP comes from the malignant cancer cells and CAFs to degrade and remodel ECM.^{47,48}

14.3 3D Cancer Modeling Tools

14.3.1 Shift From 2D Modeling to 3D Modeling: Why is it so Important?

Due to complex tumor microenvironments and poor standard preclinical models, developing oncology drugs has been successful for a few selectively active drugs. As 2D-cultured cell lines cannot precisely predict the outcomes of clinical trials, there was an inevitable shift towards 3D culture to mimic the *in vivo* tumor microenvironment (Figure 14.7).⁴⁹ Conventionally, a monolayer 2D culture is carried out in flasks or petri dishes. The advantages of 2D culture are simple culture methods, cheap maintenance fee and direct observations. However, 2D culture possesses far more disadvantages. Firstly, cells cultured in 2D flasks do not portray any *in vivo* structures of natural tissues or tumors. For instance, cell-to-cell and cell to extracellular environment communication occurring in tumors does not appear under flask culture conditions. The reason we need to observe this is that such interactions are responsible for recapitulating better tumor microenvironments that regulate tumor behaviors. Secondly, after tumor tissues are dissected from human bodies and transferred to 2D culture conditions, proliferation

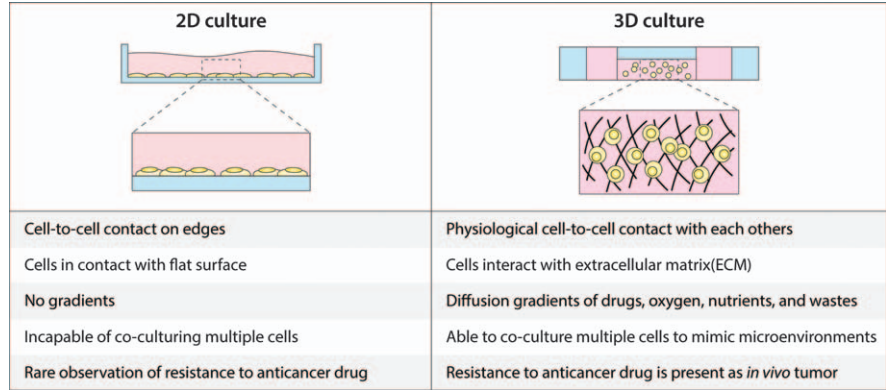


Figure 14.7 Major differences and improvements between 2D cell culture and 3D cell culture.

and differentiation are hugely different from *in vivo*. Not to mention unpredictable loss of actual phenotypes. Thirdly, 2D culture conditions in flasks are easily exposed to growth factors, oxygen, nutrients, metabolites and molecules. Tumor microenvironments *in vivo* comprise a scarcity of oxygen and nutrients, resulting in phenomena such as hypoxia and acidic conditions. Furthermore, 2D culture systems alter gene expression, topology, biochemistry and much more. Lastly, 2D culture system allows the study of only one type of cancer cell, omitting diverse tumor microenvironments.^{50–52} As a result, various 3D modeling tools such as microfluidic chips, spheroids, and organoids have been introduced and will be discussed further.

14.3.2 Microfluidic Devices

Beginning in the early-2000s, microfluidic devices, etched and molded into desired designs using silicon, glass, and polymers like PDMS, opened a new era of modeling 3-D tumor microenvironments.⁵³ Through highly delicate devices (see Figure 14.8) at micro-scale levels, as the name implies, tumor microenvironments can be recapitulated that are much more like that of *in vivo*. Primarily using PDMS and soft lithography, the microfluidic devices are very cost effective, easily fabricated, and rapidly processed.⁵⁴ Microfluidic devices offer opportunities to study cancer environments such as chemical communication, physical stimuli, and cellular microenvironments. For example, chemical communications are comprised of paracrine and autocrine signaling and EGF, VEGF cytokines from cancer cells. Physical stimuli imply the complete control over pressure, flow, gradients and quality imaging and numerical analysis.^{55–57} Within micro-scale devices, phenomena such as cancer metastasis, tumor extravasation, detection of circulating tumor cells, manipulation of flows, and high throughput drug screening can be recapitulated, offering chances to study *in vivo* cancerous characteristics in depth.

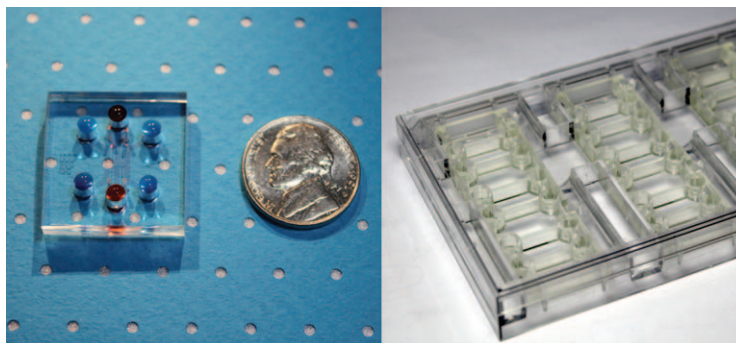


Figure 14.8 Examples of various microfluidic devices for 3D cell culture, made of PDMS (left, developed by Seok Chung (Korea University, Seoul, Korea) and Roger D. Kamm (MIT, Cambridge, MA)) or hard plastic, cyclic olefin copolymer (right, developed by AIM Biotech Pte. Ltd., Singapore).

Microfluidic models are good means to study cancer metastasis in which co-culture systems with multiple cells, complete manipulation and control over gradient, comparable scale, laminar flow in micro-channels, and mass transport governed by local diffusion can be studied. One of the microfluidic models to observe tumor behaviors is metastasis in the device.⁵⁸ During metastasis, circulating tumor cells (CTC) undergo extravasation – contacted with the endothelium, CTCs adhere to adjacent vascular walls, moving through endothelial and pericyte layers. In metastasis models between breast cancer cells and endothelium cells, the recapitulation of breast cancer extravasation was introduced. Cells used in this model were human dermal microvascular endothelial cells (hMVECs), and MDA-MB-231 (breast cancer cell line). The results clearly presented that a significant surge in endothelium permeability was observed after the introduction of breast cancer cells. Going one step further, a tri-culture system of human umbilical vein endothelial cells (HUVECs), MDA-MB-231 (breast cancer cell line), and hBM-MSCs (osteocytes) has been built in a microfluidic system with three media channels and four independent gel channels. Inside The PDMS channels were coated with PDL, collagen type 1, and Matrigel within 3 days. The results showed that cancer cells transmigrated across the endothelial monolayers such that more extravasation was induced in the osteo-differentiated environment. In addition, by adding blocking agents of CXCL5, osteoblast-secreted inflammatory chemokine, and CXCR2, breast cancer cell surface receptors, extravasation ability and rate was tested. However, blocking CXCR2 had no imminent effects on distance travelled, suggesting that other receptors may have more critical roles.

Organ on a chip is an excellent example of alternative microfluidic devices to study cancer at organ level. One of the most renowned research facilities in Harvard, the Ingber lab invented two lung cancer chips which recapitulate the small airways, air sacs, and alveoli. All these mimicked mechanisms that are responsible for oxygen and carbon dioxide exchange were cocultured

with a variety of non-small cell lung cancers. Even better, by applying actual mechanical forces that are cyclically similar to the human act of breathing, studying the actual lung environment was accomplished. The shocking importance of mimicking actual mechanical forces in the body becomes so appreciated when drugs are tested. The Ingber team discovered that when the mechanical forces of the human lungs were absent, the tyrosine kinase inhibitors known as anti-cancer drugs successfully controlled the population of the cancer. As such, organ on a chip is not only beneficial in understanding natural phenomena in the body, but it also gives us opportunities to study how tumors interact with the resided organs.

14.3.3 Tumor Spheroids

A tumor spheroid model shows great similarities to tumors in its physiology, functions, and structures (Figure 14.9). The best advantages offered by 3D cancer spheroids are the abilities to mimic *in vivo* hypoxic tumor conditions, cell-to-cell junctions, signaling, and diffusive transportation of nutrients, oxygen, metabolites, and small molecules.⁵⁹ Tumor spheroid models can be categorized into two major groups: multicellular tumor spheroids and tissue-derived tumor spheres.

In multicellular tumor spheroids, a number of methods are used; however, a main principle is to rely on cell-to-cell junctions to form tumor spheroids. By placing tumor cells on substrates that are less adhesive than tumor cells' own cell-to-cell junctions, tumor cells attaching to the surface of the substrate is avoided, allowing the formation of spheroids.⁶⁰ Usually,

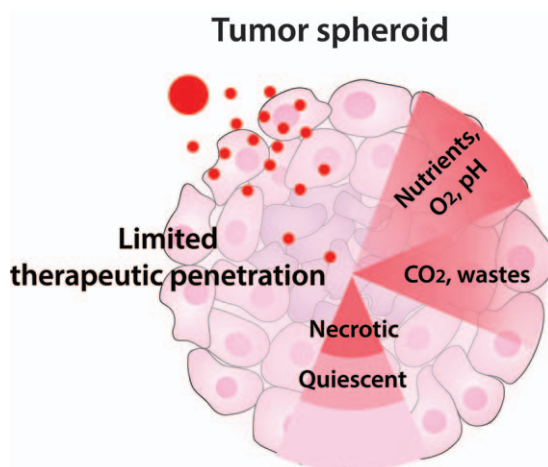


Figure 14.9 Similar features between *in vivo* tumors and tumor spheroid models. As *in vivo* tumors do, spheroid models also display three distinctive structures: Necrotic, quiescent, and proliferation. Due to structural similarities, tumor spheroids also exhibit gradients of pH, metabolic waste, nutrients, and gases.

multicellular tumor spheroids are formed within 3–4 days. Of course, the stabilization time of spheroids can vary with different kinds of cell lines. These tumor spheroid models can be promoted when covered with agar,⁶¹ polyHEMA,⁶² and agarose.⁶³ Using these so, the user should wait for substances to be dried before adding the medium, therefore promoting tumor cells to aggregate and gather in a spheroid. Furthermore, additional means such as ultra-low attachment plates and polystyrene surfaces offer tumor cells to form spheroids by offering low adhesion surfaces.⁶⁴ Through the use of the liquid coating techniques previously talked about, homogenous single multicellular tumor spheroids are produced in 96 well plates, allowing high throughput screening with the addition of robotic automation.⁶⁵

The tissue-derived tumor spheroids, which actually come from dissected cancer tissues, are far different from multicellular tumor spheroids, formed with single cells. The most well-known tissue-derived tumor spheroid is perhaps the spheroids from colon tumor tissues. These so-called colospheres are produced by slicing the tissue sample from a human body then crushing the tissue with a striated plunger from a syringe.⁶⁶ By culturing sliced tissues as spheroids, more *in vivo*-like experiments can be carried out. To further investigate tumors, tissue-derived organoids will be discussed.

14.3.4 Cancer Organoids

Starting from the most widely-used colorectal tumor, lung, gastric, brain, pancreas and other tumor organoids models are now available (Figure 14.10). The difference between spheroids and organoids is the presence of stem cells that allow simultaneous proliferation and differentiation in organoids.⁶⁷ Not to mention, tumor organoids actually represent genetical similarities to tumors harbored in patients. To further talk about organoids in depth, colorectal organoids will be mainly discussed.

Colorectal tumors are known to develop in adenomatous polyps, advanced adenomas, carcinomas *in situ*, and adenocarcinomas. In order to fully progress into malignant tumors, continual genetic mutations must occur.⁶⁸ The foremost genetic mutation would be gene adenomatous polyposis coli which controls tumor suppression. In addition to mutations in APC, mutations in the Wnt pathway are also detected. Both APC and the Wnt pathway mutations are initial mutations in colorectal tumors.^{68,69} MLH-related mutations (DNA repair), TP53 (cell-cycle regulation), and various growth factor signaling are observed with colorectal tumors. The reason why cancer stem cells within colorectal tumors are important is that cancer stem cells mainly give rise to fully grown tumors.^{68,69} Therefore, culturing single cancer stem cells can surely promote full tumor growth.

While describing the colorectal tumor organoids, the culture methods should also be discussed. Patients with adenomas and adenocarcinomas are the main models for endoscopic biopsies.⁷⁰ As one of the tumor characteristics, colon tumor organoids can grow indefinitely if a sufficient amount of growth factors and spaces are offered. While proliferating, the

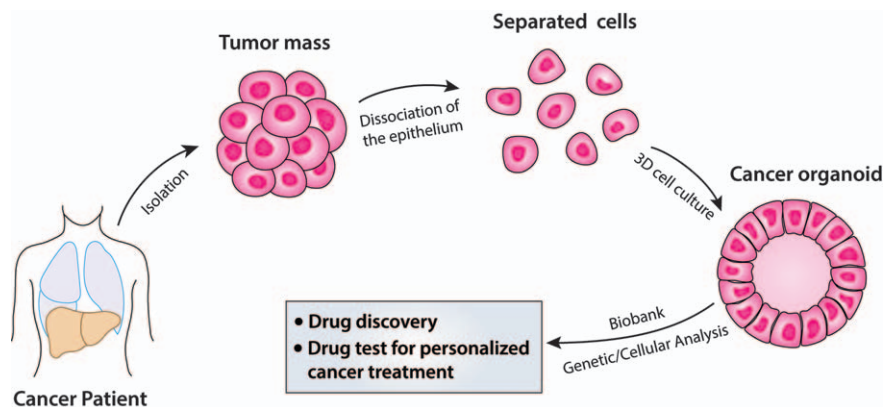


Figure 14.10 Steps to form cancer organoids and their application. Patient-derived cancer organoid models can offer various basic and clinical applications: genetic analysis, drug development, clinical response prediction in patients, and biobanks.

tumor organoids produce diverse differentiated cells homogenous to the patients, allowing much more drug resistance than conventional tumor spheroids.⁷¹ To increase diversity of tumor organoids, normal organoids are cocultured to mimic microenvironment with various stromal cells. Although all the cells come from the same patients, there is always a chance of cross contamination. Thus, specific culture conditions with pure tumor tissues are pivotal (the contamination can be easily spotted in colon tumor organoids since the boundaries are well defined). Mainly with Wnt pathway mutations, the formation of colon tumor organoids is promoted. As stated before, since the APC genes are in charge of tumor suppression, without Wnt or Wnt activator, R-spondin-1, the colon tumor organoids grow well.^{72,73} In addition, epidermal growth factor, the core factor in normal colon organoids, can promote more robust colon tumor organoids.⁷⁰ However, as the name implies, the tumor does not necessarily need all the compounds as normal organoids do. It is plausible to say that with a minimal amount of substances tumor organoids can be produced, but, in this case, the purity of the tumor tissue and the characterization of the organoids are essential. As tumor organoids such as colon organoids are easy to reproduce and proliferate without much effort, various tumor organoids are stored as biobanks throughout the world. Such a promising future for organoids has opened up a total new era of 3D tumor modeling.

14.4 Conclusion

Conventional 2D cell culture methods have given us the chance to understand fundamental biological, chemical, and molecular happenings in living cells. However, as the world of biology develops, limitations in models with monolayer cultures have arisen. The development of delicate 3D *in vitro*

models that recapitulate the tumor microenvironment and metastasis further enlightens our limited knowledge of tumors. Closely resembling human systems, 3D modeling presents new cell morphologies and expressions far improved over 2D plate cultures, leading to the inevitable transition from 2D to 3D modeling. Some fundamental results from recent research are: *in vivo*-like formation of cancer cell clusters, metastasis, proliferation, hypoxic conditions in the center, and the release of numerous tumor-promoting factors. These all add up to offer promising methods for testing drug efficacy. However, this does not mean that 3D modeling is perfect for studying tumors. Known problems in 3D modeling are: multicellular tumor spheroids do not comprise all types of cells, and the components of the extracellular matrix do not represent the true tumor environment. The synthetic scaffolds used in microfluidic devices and spheroids are not and will never be natural matrices. Furthermore, even if 3D devices can mimic the happenings occurring in the human body, the depth of recapitulation is simple and straightforward. However, history is not written in a single day. All the steps that 3D modeling is going through are the paths that must be taken that will lead into definitive tumor microenvironment modeling in the future. Finally, all these improved biomimetic models offer a bridge between 2D systems and *in vivo* models, delineating molecular mechanisms underlying tumor growth and progression and drug action.

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CHAPTER 15

3D Tissue Models for Toxicology

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15.1 Introduction

In the development of chemicals, be it for pharmaceutical, agricultural, cosmetic, or other use, it is necessary to study the adverse effects the compounds may have on living organisms. This field of research is known as toxicology and underpins the successful development and marketing of chemicals. Toxicology research often begins with simple 2D cultures of human cells, which can be screened with chemicals and analysed in a high-throughput manner. From this, highly toxic compounds can be discarded and leads analysed further. These tests, however, are often focussed on narrow outputs of interest, meaning their relevance to human-scale toxicity is limited. Later-stage chemical development requires more in-depth phenotypic toxicology data, and this is often procured from animal research. In 2016, 13.5% of all animal research in the UK was for regulatory satisfaction, primarily in toxicity research.¹ *In vivo* research provides more detailed toxicity outcomes, but the use of animals obviously has several drawbacks ethically, economically, and biologically. Using animal models to relate toxicity to humans relies on assumptions that the biological systems will be sufficiently

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alike to extrapolate results. As well as this, reducing the use, and improving the welfare of research animal animals is essential in the future of scientific research.² Taken together, it becomes clear an adjunct or even alternative to current toxicity methods is required to improve chemical development and animal welfare. Interest has grown in the use of biofabricated 3D tissue models to supplement toxicity studies in chemical development.

In recent years the field of biofabrication has grown rapidly. Defined as “the automated generation of biologically functional products with structural organisation from living cells, bioactive molecules, biomaterials, cell aggregates such as micro-tissues, or hybrid cell-material constructs, through bioprinting or bioassembly and subsequent tissue maturation processes”,³ biofabrication has the potential to generate human tissue-like structures ‘from scratch’. These fabricated tissues can be utilised during chemical development to assess the effects of compounds on various human cell and tissue types. By drawing on human cells and tissue architecture, biofabricated tissues could provide more comprehensive readouts and bear more biological relevance than both 2D cell and animal toxicity models. The result of this would be reduced attrition in chemical development, and improved animal welfare.

A comparison of traditional 2D monolayers, animal research, and novel biofabricated tissue and microfluidic/organ-on-a-chip models for toxicology research is summarised below (Table 15.1). Several factors are necessary to consider when choosing a suitable research model. High-throughput research, in the case of high content drug screening, is much more amenable to 2D monolayers than both animal research and most biofabricated tissues, owing to speed of production, and low cost of a single unit.⁴ New microfluidic platforms however can find a middle ground between simple 2D monolayers and biofabricated tissue and organs.⁵ Determining the mechanism of toxicity of candidate compounds can be crucial in its development. The ability to do so depends deeply on the model used. In the case of 2D monolayers only mono-cellular effects can be observed.⁶ However, in animal

Table 15.1 Comparison of primary toxicology models “+++” strong capability, “++” middling capability, “+” low capability, “–” no capability.

	Model			
	2D monolayer	Animal research	Biofabricated tissue	Microfluidic/organ-on-a-chip
High-throughput	+++	–	+	++
Detailed mechanism of toxicity	+	+++	+++	–
Relevance to endogenous tissue	+	–	+++	++
Scalable	+++	–	+++	+
Amenable to histopathology	–	+++	+++	–
Can observe systemic effects	–	+++	++	+++

models⁷ and biofabricated tissues toxicity caused by interactions between various cell types in a tissue can be analysed. Relevancy to native tissue, and indeed humans, is essential when analysing toxicity in models, as certain assumptions and extrapolations will always be necessary. In the case of 2D cultures, simplicity means you will not see toxic effects that rely on other factors, such as immune or supporting cells.⁸ With animal models, the inherent biological differences means they are never true predictors of toxicity for humans.⁹ Biofabricated tissue and organs-on-a-chip aim to recapitulate qualities and characteristics of native tissues, and so will be more predictive, especially when utilising human cells.

Scalability of the model can refer to both the number of units that can be produced feasibly (relating to high-throughput) but also the size of model that can be produced. As discussed, simple cultures are easily scaled up, but animal models are expensive to house. Biofabricated tissue can be produced in small scale (high-throughput) or as larger tissue or even organs (mid/low-throughput) for use in both initial drug screening, as well as in-depth toxicological analysis. Microfluidic cultures are limited in the size that can be produced, but new manufacturing technology, such as those utilising polydimethylsiloxane (PDMS) allows quick moulding and casting of microfluidic devices.¹⁰ Histopathological analysis, the study of disease on tissue using microscopy of a sample,¹¹ is a key tool in toxicology research as it gives information on the effects of the target compound on tissue and organ before use in humans.¹² This technique is inherently unusable with 2D monolayers, and is used with animal models.¹³ Histology is also possible on biofabricated tissue of sufficient size,¹⁴ improving its capability as a toxicological tool. Microfluidic cultures are often encapsulated in the device, making them difficult to extract for further characterisation. However, when considering systemic toxic effects of a compound, microfluidic devices are paving the way for an *in vitro* method to assess interactions of potential toxic compounds on various tissue types. In multi-organ-on-a-chip technology, tissues of different organs are cultured and perfused in a circuit, that allows metabolites, chemokines and other signals to elicit effects on other organs, as it would *in vivo*.¹⁵ This kind of systemic analysis is currently only undertaken in animal models, but biofabricated models that source tissue from different organs have also been developed that could be used.¹⁶

This chapter will elucidate the current state of biofabricated 3D models for toxicology testing in several tissues of interest: skin, liver, kidney and heart. It will also detail the necessary steps for *in situ* biofabricated tissue research to take before it can be used as a viable alternative in toxicology research.

15.2 3D Skin Models for Toxicology

As the largest organ, as well as the primary barrier for entry to the body, adverse effects to the skin are critical in chemical development and toxicology. The EU Cosmetics Directive also restricts the use of animal testing during cosmetic development, driving the need for accurate *in vitro* models

for toxicity testing.¹⁷ Historically, the Draize test¹⁸ tested dermatotoxicity by application of the desired chemical to the skin of, most commonly, a rabbit, and qualitative observation of the effect. This does, however, give no insight to the mechanism of toxicity or quantitative or easily comparable results. With aims to reduce animal research use, particularly in cosmetics, *in vitro* alternatives have been sought. Typically, primary or keratinocyte-derived cell lines have been used to study dermatotoxicology *in vitro*, as keratinocytes compose the bulk of epidermal tissue. However, these simple 2D cell models lack many key endogenous features of skin which may influence the robustness of any toxicity tests. Often there is no 3D architecture, which means the typical dual-layer of epidermis and dermis is lost. There is also a lack of supporting cells, such as Langerhans cells and melanocytes. Improving the efficacy of human skin equivalents (HSEs) has been made possible by better mimicking of endogenous tissue. Skin is unique in that validated 3D *in vitro* alternatives for toxicology already exist and are industrially available. SkinEthic,¹⁹ MatTek,²⁰ and CellSystems²¹ all market HSEs of varying complexity, with MatTek's EpidermFT and CellSystems' Skin Tests containing a co-culture of keratinocytes and fibroblasts in a multi-layered structure.²² Key tissue characteristics are still lacking from these models, however. The amenability of biofabrication techniques could allow these complex structures to be reconstructed *in vitro*.

Biofabrication techniques such as bioprinting have shown the ability to generate a 3D layered co-culture of fibroblasts and keratinocytes,^{23,24} as well as tri-cultures containing additional adipose-derived cells to recapitulate the hypodermis^{25,26} (Figure 15.1). The automated nature of bioprinting makes it ideal for the generation of many tissue structures for high-throughput analysis. In addition, inclusion of adipose cells can improve the prediction of toxicity to what would be expected in the body.

As well as being a barrier to xenobiotic materials, metabolism of chemicals is also a known function of the skin. A 3D fabricated model of skin has been utilised to examine Phase 1 and Phase 2 enzyme activity, and it was shown that 3D HSEs have a more similar detoxification capability to natural skin than simple 2D monolayers, making them more suitable for dermatotoxicity studies.^{27,28}

Much of the validation of these artificial skin structures has been with *in vivo* application, with little focus on toxicological comparison to current gold standards or primary skin tissue, but this is likely to change as the field of biofabrication matures. With large companies showing keen interest in bioprinting of skin for research, such as l'Oréal's partnership with bioprinting firm Poietis,²⁹ it is clear there is a continued need for a suitable, biofabricated 3D skin model for industrial use.

15.3 3D Liver Models for Toxicity

As the primary site of xenobiotic metabolism in the body, the liver is an organ with a high chance of exposure to toxic compounds or their resultant

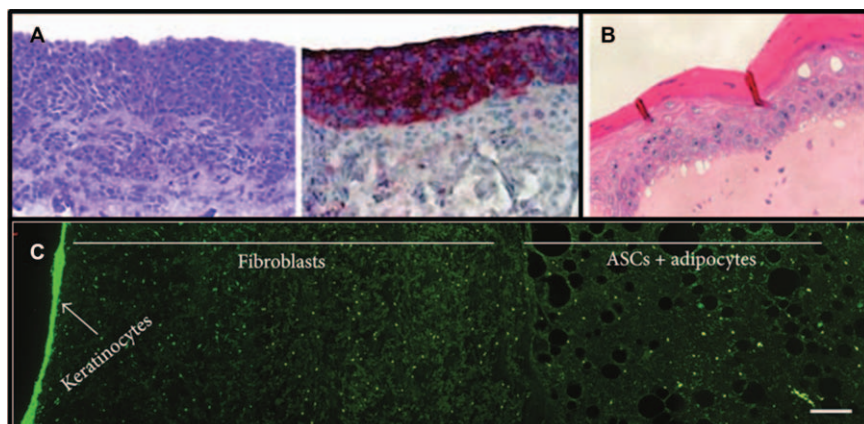


Figure 15.1 Biofabricated skin tissue. A: These two images show a co-culture of human fibroblasts and keratinocytes, laser printed into the biomimetic, bi-layered structure of native skin. L shows haematoxylin and eosin (H&E) staining of the structure, R immunoperoxidase of cytokeratin 14 in reddish-brown.²³ B: Shows H&E staining of biofabricated tri-culture of human fibroblasts, keratinocytes, and also adipose cells, incorporating additional subdermal layer of skin tissue.²⁶ C: Shows fluorescent green stain of another tri-layered culture, visualizing the defined lamination of tissue structure.²⁵

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metabolites. Indeed, drug-induced liver damage is the primary reason for approved drugs being pulled from market.³⁰ When studying liver toxicology, the ability of the liver model to metabolise drugs is another factor that must be considered, as well as the adverse effects the metabolites may produce. Biotransformation of drugs and other xenobiotics is primarily achieved through the cytochrome P450 system and oxidation,³¹ and these metabolites can be either more or less toxic than the original compound. A liver toxicity model must have the ability to metabolise drugs akin to endogenous liver in order to produce relevant results. This, in turn, is dependent on recapitulating key characteristics from the tissue: 3D tissue architecture; co-culture with supporting cells; cell-material interactions; as well as biomechanical forces acting upon the liver, *i.e.* vascular shear stress.

Human liver cell-based toxicity models are historically simple and lose many key aspects of liver tissue. Primary hepatocytes cells are prone to dedifferentiation and reduction in function very soon after isolation. The scarcity of primary human hepatocytes also limits their use. Additionally, the non-parenchymal cells of the liver (including Kupffer cells, stellate cells, endothelial cells, and others) have known roles in hepatotoxicity,⁸ so their absence from toxicity models reduces the models predictive capacity.

Biofabrication techniques have been employed to produce 3D models for hepatotoxic research that attempt to overcome the limitations of other models. Cell source is an essential consideration when generating toxicity models, and for liver models there are several cell source possibilities. Primary human cells have the greatest relevance to native hepatocytes but maintain endogenous function only briefly after *in vitro* isolation. Animal-derived primary cells, particularly rat have also been used to generate *in vitro* models.³² Immortalized hepatic lines, primarily HepG2³³ and HepaRG³⁴ have also been used to assess hepatotoxicity of drugs, but as they are immortalized, can harbour aberrant phenotypes and chromosomal abnormalities. Stem cell-derived hepatocytes could provide a theoretically limitless supply of hepatocytes,³⁵ from a varied genetic background, making it ideal for toxicology studies.

Incorporating the primary human hepatocytes into a 3D culture model has shown to improve their longevity, as well as metabolic function.^{32,36} In particular, improved acetaminophen-induced hepatotoxicity sensitivity was seen in 3D culture over 2D, correlating with an increase in the P450 isoform, CYP2E1.³⁷ The comparative relevance of the HepG2 cell line to primary human hepatocytes was similarly improved by culturing HepG2 cells in Matrigel.³³ HepG2s cultured in this way self-organised into spheroids and displayed mature hepatocyte features, including polarity, hepatic gene expression and cytochrome activity.

Translation of hepatocyte culture from 2D to 3D has been shown to improve the relevancy of the model to endogenous liver tissue alone, but by incorporating more features of the native liver environment, *in vitro* models have become increasingly intricate and biomimetic. Shear stress, experienced by hepatocytes through fenestrated sinusoidal endothelial cells in the liver,³⁸ is a fundamental biophysical force acting upon the tissue, which has been recapitulated *in vitro*. A perfused 3D culture system of primary rat hepatocytes displayed similar IC₅₀ values of 5 model drugs to *in vivo* LD₅₀ values.³⁹ Likewise, pluripotent stem cell-derived drug sensitivity was seen to be significantly altered under dynamic culture.⁴⁰

Furthermore, combination of 3D architecture, dynamic culture, and multi-cellular culture of hepatocytes and supporting cells has been achieved, to produce highly biomimetic 3D models. Liver tissue is organised into characteristic hexagonal lobules,⁴¹ and, by utilising 3D bioprinting, researchers have recreated this hexagonal architecture with defined 3D patterning of induced pluripotent stem cell (iPSC)-derived hepatocytes, as well as supporting endothelial and mesenchymal cells.⁴² These biofabricated tissue structures displayed improved morphology, phenotype, metabolism and CYP activity, and gene expression over 2D monolayer and a 3D monoculture of hepatocytes. Similarly when primary rat hepatocytes were co-cultured with endothelial cells in a microfluidic platform to form an artificial “liver sinusoid on a chip”, the cultured hepatocytes displayed improved longevity and function over 2D alternatives.⁴³ A further study fabricated a 3D hepatic model that comprised primary rat hepatocytes,

stellate cells, Kupffer cells, and endothelial cells. The resultant tissue preserved function for up to 3 months, including key protein secretion, as well as cytochrome P450 inducibility, bile canaliculi formation and response to inflammatory signals. Of particular note, when challenged with toxins known to induce idiosyncratic hepatotoxicity, this 3D model was a better indicator of *in vivo* toxicity than standard 2D monolayers.⁴⁴

Another microfluidically perfused co-culture system, which utilised the HepaRG cell line as well as supporting human non-parenchymal cells, attempted to further recapitulate endogenous architecture by creating an artificial space of Disse in the 3D culture *via* a suspended membrane.⁴⁵ Particular to this work was the inclusion of sensitive oxygen monitors to record oxygen intake by the cultured cells. This is relevant for xenobiotic metabolism of the liver, as there is distinct zonation of the liver lobule defined by the oxygen concentration available, known as the acinus.⁴¹ Dependent on which zone a hepatocyte is present, it will carry out specific metabolic processes: high oxygen availability, or periportal hepatocytes are involved in processes such as gluconeogenesis; whereas low oxygen or perivenous hepatocytes have higher cytochrome P450 activity.⁴⁶ Given the variety in phenotypes across the acinus, it becomes clear 3D liver models that incorporate oxygen gradients and measurements, as the one above, will improve its predictability for hepatotoxicity.

Primary human cells have also been used in bioprinting 3D models, with patient hepatocytes, stellate cells, and endothelial cells used to produce a bioprinted tissue model for drug-induced toxicity.⁴⁷ Sustained hepatic activity over 4 weeks of culture was seen, and its ability to assess hepatotoxicity of a drug, Trovafloxacin whose toxicity was not assessed in earlier pre-clinical models, was tested. It was shown that this biofabricated tissue, formed of primary human liver and supporting cells, displayed a dose-dependent hepatotoxicity at clinically relevant concentrations of the drug, making it suitable for assessing drug-induced liver injury. A similar strategy was used previously with scaffold-free 3D microtissue formation: primary human hepatocytes, Kupffer cells and stellate cells were formed into spheroidal microtissue by gravity, and the resultant 3D model showed improved longevity and function over 5 weeks.⁴⁸ As well as this, inclusion of Kupffer cells allowed assessment of immune system-mediated effects in hepatotoxicity, which was probed with the inclusion of lipopolysaccharide to activate the macrophages. A summary of different biofabricated liver models are summarised below (Figure 15.2).

3D modelling of liver tissue is one of the fastest moving fields of biofabrication, and this is a boon for toxicology models. Already, biofabricated systems have incorporated several cell types into a tissue-like architecture,^{42,45} some with perfused culture to mimic the biophysical forces of the liver. Given the liver's key role in homeostasis, as well as xenobiotic metabolism, it is a key target for bioengineers developing toxicology models. Not only must metabolic processes be assessed in these models, such as cytochrome activity and prodrug metabolism, but also typical cytotoxicity

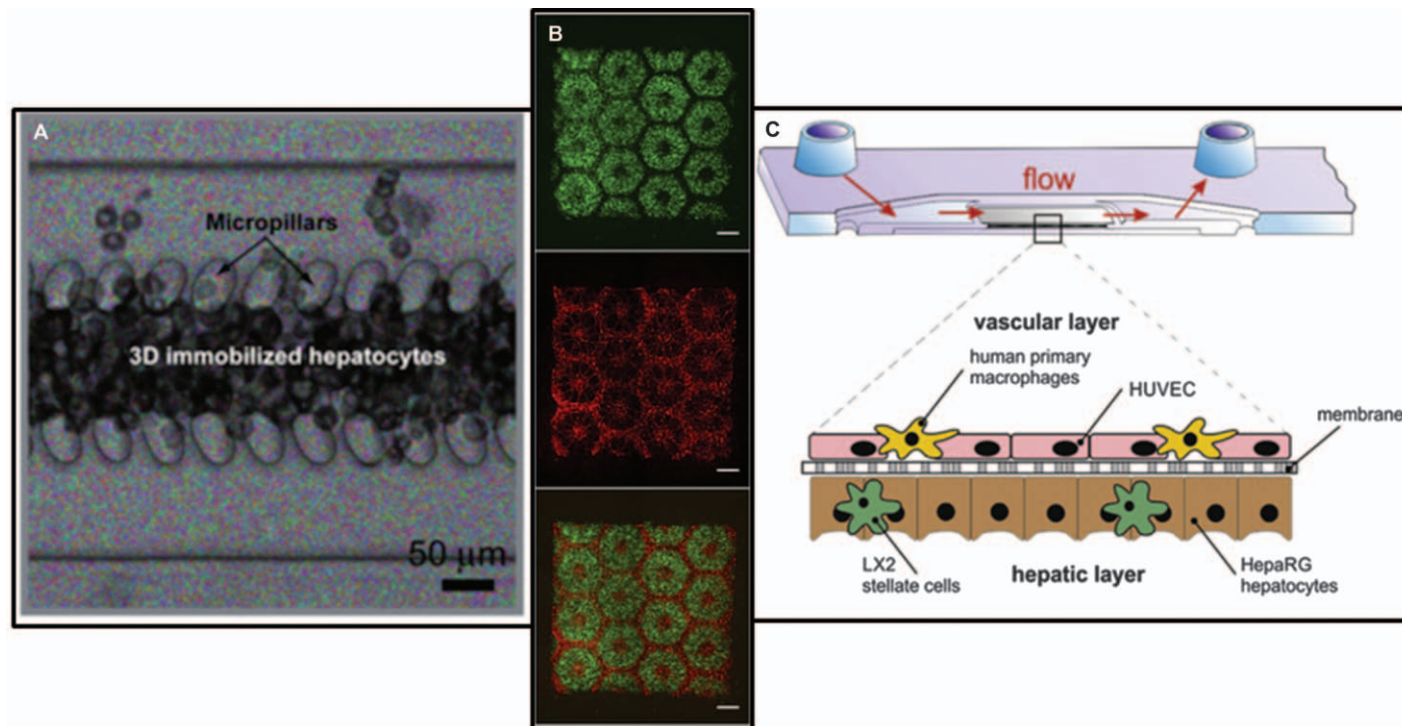


Figure 15.2 A: 3D cultured primary rat hepatocytes in a microfluidic device. The culture chamber is separated by fenestrated micropillars from the perfused region, mimicking shear stresses experiences in the liver.³⁹ B: Fluorescent stains of a hexagonal 3D culture model fabricated by bioprinting. Green fluorescent highlights IPSC-derived hepatocytes, with red highlighting supporting endothelial and mesenchymal cells. The placement shows structural similarity to the hexagonal lobule of native liver tissue.⁴² C: Another schematic for a microfluidic device which aims to mimic the liver sinusoid. However in this instance endothelial, macrophage, stellate and hepatocyte cells are to be incorporated in the model, which will predict any interaction between different cell types that may occur *in vivo*.⁴⁵ Part A reproduced from ref. 39 with permission from the Royal Society of Chemistry. Part B reproduced from ref. 42 with permission from PNAS. Part C reproduced from ref. 45 with permission from Elsevier, Copyright 2015.

caused by target compounds. Future models may include intrinsic vascularization, oxygen gradients to mimic the acinus, or innervation, to bring them even closer to true tissue-like constructs.

15.4 3D Kidney Models for Toxicity

The kidney receives a substantial portion of cardiac output and is a primary site of excretion from the body. Together this results in high exposure of kidney tissue to potentially toxic xenobiotics, which can produce nephrotoxicity.⁴⁹ While only 7% of new drugs fail during development due to detected nephrotoxicity, new drugs are the cause of 30–50% of all acute kidney injuries.⁵⁰ This makes a clear case that improved models are required to detect nephrotoxicity during drug development. Adequate models of the kidney are difficult to devise however, given the intricate architecture of the kidney and nephron. Both 2D cell layers⁵¹ and animal models⁵² have been used to assess nephrotoxicity, but are limited by the factors discussed previously for other tissue. Biofabricated kidney tissue have the potential to improve upon these models to better predict nephrotoxicity or determine suitable biomarkers to monitor it.

An immortalized human renal cortical epithelial cell line was used to generate a 3D kidney model in a Matrigel and collagen hydrogel.⁵³ The system showed it was able to use current pre-clinical kidney injury biomarkers to determine toxicity. Importantly, the model was also amenable to long-term (2 week) dosing of known nephrotoxic compounds, making it a stronger tool for repeat-dose studies than 2D equivalents.

Whole proximal tubule isolation from mice has allowed the fabrication of kidney-organoids *in vitro*.⁵⁴ This protocol used a combination of the harvested, intact tubules with a supportive hyaluronic acid and polyethylene glycol diacrylate scaffold to sustain the 3D architecture of the tubule. This 3D organoid culture has shown improved consistency with *in vivo* nephrotoxicity *versus* 2D immortalized kidney cell lines,⁵⁵ and has been used as a tool to assess nephrotoxicity of known toxic agents⁵⁴ as well as nanoparticles, which are gaining traction as pharmaceutical or medical imaging carriers.⁵⁶ Results showed similar nephrotoxicity seen in rodent models.

The generation of pluripotent stem cell-derived kidney organoids holds promise for toxicological research, as it does not rely on animal use, or genetically invariable cell lines. By guiding pluripotent stem cells through early development stages, it is possible to produce kidney organoids, suitable for toxicology testing.⁵⁷

Fabrication of whole proximal tubules has been achieved by bioprinting a tubule cast of ECM materials before seeding with immortalized human proximal tubule epithelial cells (PTECs), (Figure 15.3). The resultant artificial tubule is composed of densely packed PTECs that display stronger endogenous phenotype and morphology compared to 2D controls. As well as this, they react in a dose-dependent manner to nephrotoxic agent, Cyclosporine A, with the epithelial barrier of the tubule itself being disrupted depending on toxin dose.

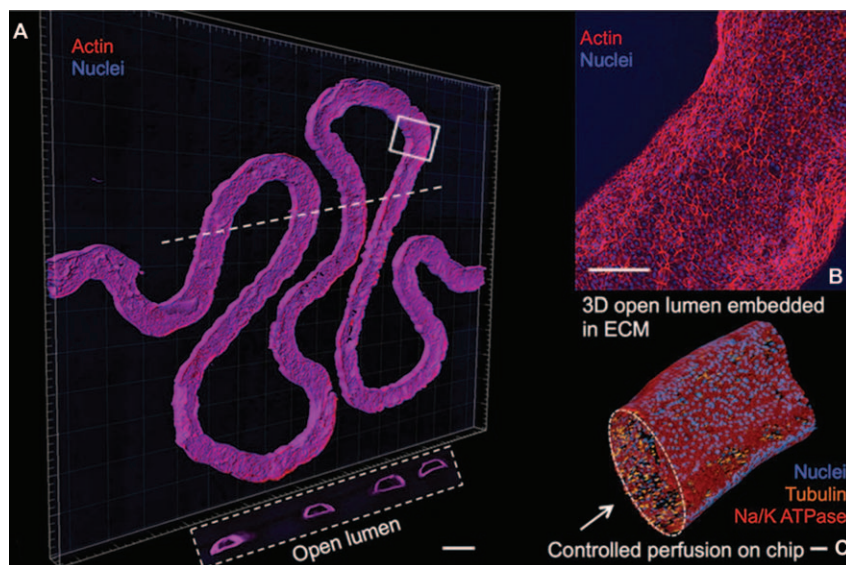


Figure 15.3 Bioprinted proximal tubule comprising of immortalized, human proximal tubule epithelial cells (PTECs). A shows the gross structure of the tubule, with a cross-section showing the open lumen that was perfused throughout culture. B shows the densely packed nature of the tubule, comprised of PTECs, whereas C shows a close-up of the open lumen for perfusion, as well as expression of tubulin and Na/K ATPase, PTEC markers. Reproduced from ref. 74 under the terms of the CC BY 4.0 licence, <https://creativecommons.org/licenses/by/4.0/>, <http://dx.doi.org/10.1038/srep34845>, Copyright The Authors.

As with the liver, it is important to assess both the metabolic as well as cytotoxic aspects of kidney toxicity. Cytochrome activity in the kidney could affect target compound metabolism, and clinical biomarkers for kidney damage must also ideally be utilised while developing *in vitro* models. However, the kidney has a very complex architecture. With around a million of nephrons in a kidney, organ-scale kidney formation is likely still a far way off. However, as shown above, a perfusable proximal tubule has been formed by bioprinting, and the main steps between that and organ-level printing are scalability and vascularization.

15.5 3D Cardiac Models for Toxicity

Cardiac toxicity was the leading cause of drug withdrawal from 1975–2007, above even hepatotoxicity.⁵⁸ Given the systemic nature of the cardiovascular system, the finely tuned function of the heart, as well as its poor regenerative ability,⁵⁹ cardiotoxicity that has not been properly assessed preclinically is also a serious concern for patient health. For toxicology, primary animal cells and immortalized cell lines are used to assess direct cytotoxicity of compounds, whereas animal models can determine systemic effects of

drugs.⁶⁰ Animal models for cardiotoxicity are far from perfect – heart rates are many times faster than humans, and they lack the human-ether-a-gogo-related gene (hERG), which is a site of known cardiotoxicity for many drugs.⁶¹

The production of cardiomyocyte-like cells from human pluripotent-cells⁶² has allowed a ready source of human cells for biofabrication, to design and test cardiotoxicity in a biomimetic model that is amenable to toxicology screening. Alone, human embryonic stem cell (hESC)-derived cardiomyocytes were able to assess cardiotoxicity of many clinical compounds, by assessing the electrophysiological effect that was produced.⁶³ Likewise, the cardiotoxic mechanism of common chemotherapeutic Doxorubicin was modelled using iPSC-derived cardiomyocytes.⁶ However it is important to consider that ESC and iPSC-derived cardiomyocytes harbour a fetal phenotype, and may require additional environmental cues to promote full maturation to a relevant mature state.⁶¹

Use of a microfluidic device has allowed bioengineers to fabricate artificial heart ventricles, whose contraction can be accurately measured and used as a tool to predict contractility stresses.⁶⁴ Isoproterenol, a clinical bronchodilator, showed dose-dependent contraction in the system. The small microfluidic device is amenable to scale-up, making it viable as a model for compounds effect on contractility.

The “Biowire” technology aims to improve the maturity of stem cell-derived cardiomyocytes by utilising 3D culture architecture with electrical stimulation.⁶⁵ Cardiomyocyte cultured on this surface showed mature cardiomyocyte characteristics, such as improved Ca^{2+} handling in response to drug stimulation, such as caffeine.

As well as improving the biology, it is important that the model has a sufficient platform to record the effects of assessed compounds, especially in cardiac tissue where significant effects are seen electro-physiologically. This has been achieved by researchers by utilising 3D bioprinting to construct a culture platform that can aid anisotropy of cardiomyocytes, as well as provide non-invasive, electronic readouts of contractility of the cultured cells (Figure 15.4).⁶⁶

The company Cyprotex, specializing in toxicology and ADME services, produce a 3D cardiac model.⁶⁷ The model utilises a co-culture of iPSC-derived cardiomyocytes, as well as supportive cardiac endothelial cells and fibroblasts. Their model can determine functional as well as structural damage caused by candidate compounds. Readouts include mitochondrial stability, cellular ATP levels, and calcium dys-homeostasis, which have all been identified as cardiotoxic effects.⁶⁸ The role of supporting cells in cardiotoxicity is known⁶⁹ so the tri-culture of the model also ensures cardiotoxic events that involve these non-parenchymal cells are also observed.

Cardiotoxicity is a constant concern for healthcare providers, especially with known chemotherapeutics such as cisplatin⁷⁰ and mitomycin.⁷¹ More predictive models for cardiotoxicity will not only allow faster and safer development of new therapies which may not produce these side effects, the

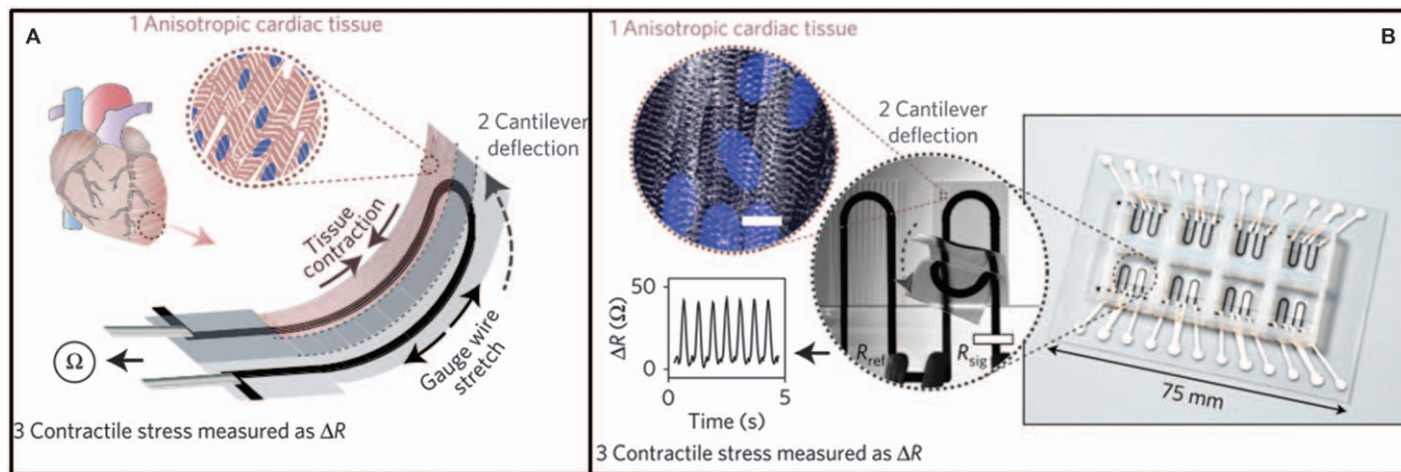


Figure 15.4 Device for culturing and measurement of iPSC-derived cardiomyocytes contractility.⁶⁶ A shows the depiction and design of the construct. Cardiomyocytes are grown on the platform to promote anisotropy, and alongside a cantilever which in turn is used to measure contractility of the cells. B shows the final printed product with aligned cardiomyocytes. Contractile stress can be measured as an output and can be related to cardiotoxicity of the target compound. Reproduced from ref. 66 with permission from Springer Nature, Copyright © 2016, Springer Nature.

models can also be used as tools to understand mechanism of toxicity and better plan treatment strategies.⁶ Modelling of cardiac tissue must measure both cytotoxicity brought on by the target compounds, as well as functional loss: primarily in contraction efficiency.

15.6 Conclusions and Future Outlook

Drug attrition and patient well-being can both be helped immensely by predictive pre-clinical toxicology models. Current 2D cell monolayers, and animal models both fall short in this regard for reasons described above. Technological advancement means biofabrication seems poised to deliver tissue-like models for toxicology studies of various organs in the near future. Techniques such as bioprinting allow generation of tissue architecture that is much more analogous to endogenous tissue than standard 2D cell culture. Bioprinting can: include multiple cells into one structure, promoting cell-cell interactions which may be crucial in toxicity; produce 3D architecture, improving cell phenotype; allow fabrication of devices which can be perfused to reintroduce biophysical forces that are typical for tissue; and are scalable. The flexibility which biofabrication presents in its scalability is one of its greatest strengths: one can either produce very small structures, for high-throughput screening; or larger, more complex tissue structures which are amenable to more in-depth analysis on the mechanism of toxicity, such as histopathology, as well as large scale-omics readouts.

Endpoints for toxicity vary depending on the tissue being assessed. In the tissue examples discussed here, they include: absorption of compounds through the skin; metabolism of the compounds in the cytochrome system of the liver; metabolism in the kidney and excretion of clinical biomarkers; and electrophysiological and contractile responses from cardiac tissue are all crucial in predictive monitoring of tissue toxicity, alongside typical cytotoxicity from the target compound. These effects can often be overlooked, or simply absent in simpler systems, where the effect is dependent on cell-cell interaction. Biofabrication offers solutions to these by allowing bespoke culture devices or platforms to meet the needs of each tissue, as has been shown with cardiac contractility in particular,⁶⁶ and the flexibility to design the structure and include various cell types.

While still debatably in its infancy, biofabrication of models for toxicology has the potential to vastly improve drug and chemical development, as well as patient health and well-being. However, few biofabricated models are currently used to assess toxicity. In the next few years we will hopefully see improvement biologically and technically in the field of biofabrication. This will allow more complex tissue formation, by improving bioprinting resolution and optimising bio-inks used therein. Vascularization is the key to developing larger, organ-like structures by biofabrication, and this may soon be possible with new technology and techniques.⁷² Multi-organ-on-a-chip or linked biofabricated tissues may soon be used to also study systemic effects of desired chemicals, predicting idiosyncratic or

off-targets more readily than current toxicity models. Importantly, collaboration and communication are important between various fields of research and regulation to improve current models and integrate new ones in chemical development. As discussed in a review paper from the National Centre for Replacement, Refinement and Reduction of Animals in Research (NC3Rs),⁷³ engineers have the tools to solve problems they may not know life scientists and chemists have, and an interdisciplinary and multidisciplinary approach will be essential to tackle the challenges in creating realistic 3D tissue models. Likewise, interactions between academics developing novel platforms and industry who can utilise them on a larger scale is key for translation. Robust characterisation and validation of new toxicology models is also imperative for their acceptance by regulatory bodies such as the FDA, or EMA, and subsequent utilisation by industry.

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CHAPTER 16

Ethics of Using Human Cells/ Tissues for 3D Tissue Models

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16.1 Introduction

The use of animal models as human surrogates is limited in the development of safe and effective new drugs or treatments in research and the pharmaceutical industry due to the low similarity and ability of these models to predict the behavior and disease pathology in humans, the feasibility of procedures, and ethical concerns.^{1–5} A comparison of the bioavailability of a range of drugs in humans with animals, rodents, dogs, and even primates has revealed a very poor level of correlation.⁶ *In vitro* models using human cells that emulate human biology have been developed to overcome these problems in the discovery of new drugs or the development of more efficacious drugs by obtaining a better understanding of human disease pathology.⁷

Two-dimensional (2D) culture systems are the most common and traditional models used for *in vitro* research. 2D culture models have contributed

to significant developments and advances in biological research, but these models have an intrinsic limitation in validating studies of *in vivo* biology. Three-dimensional (3D) models provide a cellular microenvironment that more closely mimics the microenvironment observed in native tissues. 3D cell or tissue culture models offer an alternative to natural tissue models, where many types of cells are induced to form tissue-like constructs, often using hydrogels to provide structural support for the growing tissue. These features are critical for drug testing since environmental cues have profound effects on the properties, behaviors, and functions of cells, which may affect cellular responses to drugs.^{8–14} Therefore, 3D models of human cells or human tissues offer a platform for high-throughput and systemic experimentation, reducing the need for animals and permitting a more straightforward understanding of cause and effect in drug safety and efficacy assessments. Moreover, 3D tissue models may replace human tissues and even organs in the future, thereby saving hundreds and thousands of lives.¹⁵

Although the attraction of human studies is the wide range of functions that can be studied, several ethical and policy issues related to informed consent for donation, property, distribution of profits, and quality and safety in the procurement of samples exist.¹⁶ Because the significance of biological materials varies among individuals and groups, researchers must assess the ethics of research involving these materials, with an awareness of and sensitivity to the known values, beliefs and attitudes of the individuals from whom the materials originated.

3D bioprinting is a technology used to produce tissues or organs by laminating living cells in a desired shape or pattern. Currently, most skin, organs, and bone grafts are donated. In the case of grafting, a suitable tissue or organ is difficult to obtain. Even if grafting occurs, many problems are observed due to autoimmune reactions. Studies on various artificial alternatives, such as artificial bone, dental prosthesis, artificial blood vessel, artificial skin, artificial organ, and biochips using biodegradable and biocompatible polymer materials in tissue engineering and scaffolding, are actively conducted to overcome these problems. Advances in 3D bioprinting technology will not only improve medical technology but also provide tissue, organs, and bones for transplantation into humans.^{17–20} In medicine, an organ can be produced in preparation for surgery. When developing a new drug, the efficacy and side effects of the drug can easily be confirmed through artificial non-human organs, and the impact can easily be studied.^{21,22}

Cells or tissues are the most important components in 3D bioprinting and printed products. The type of cell determines the characteristics of the 3D-printed human tissue or organ. Hence, the ethical aspects of the use of human cells/tissues, including stem cells, must be considered. Here, we discuss the ethical issues related to the source of cells, the donation of cells, and the clinical trials, which are important points to be debated in the field of 3D human tissue engineering.

16.2 Ethical Aspects of Cells

The field of human 3D tissue engineering is evolving rapidly, and some engineered 3D tissues are now ready for clinical trials. Although these engineered 3D tissues are now entering the clinical testing phase, the issues surrounding the use of human biological materials (cells and tissues) have not been commensurately addressed.^{23–25} Three clusters of ethical issues are associated with the development and clinical testing of engineered 3D human tissues: issues related to (1) the source of cells, (2) the donation of cells, and (3) the clinical translation.

16.2.1 Ethics Related to the Source of Cells

Cells are important factors that determine the characteristics of the bioengineered 3D tissue or organ. Stem cells have been frequently used in modeling 3D tissue systems, including 3D-bioprinted tissues or spheroids, due to their high proliferative capacity and ability to differentiate into multiple lineages. Stem cells exhibit significant potential for the development of bioengineered 3D tissues. Hence, the ethical issues related to the source of human stem cells are considerable issues affecting the use of cells in modeling 3D tissue systems.

16.2.1.1 Human Embryonic Stem Cells (hESCs)

De Vries *et al.* reviewed and listed approximately ten ethical issues concerning tissue engineering that are most frequently mentioned or discussed in the scientific literature.²⁶ The dominant ethical issue concerns the use of human embryonic stem cells (hESCs) in tissue engineering.^{26–30} The hESCs derived from the ‘inner cell mass’ of blastocyst stage embryos can form all somatic tissues, suggesting that hESCs are specialized cells useful for the treatment of a number of human diseases and the development of 3D tissue models for testing new drugs. However, the issues surrounding the application of hESCs are moral and normative matters. A number of individuals have proposed that an individual’s life begins at fertilization and therefore an embryo is a person. From this perspective, the removal of the inner cell mass from a blastocyst to derive an embryonic stem cell line is not morally acceptable. The hESCs are not the only cells that are considered morally and normatively problematic regarding the ethical issues with the development 3D tissue systems. Ethical arguments on the use of fetal cells^{31–35} and embryonic germ cells^{36–38} still exist because of the primary source of these cells, which may include tissues from induced or elective abortions, a morally controversial intervention. Therefore, researchers must consider the ethics related to the source of stem cells at an early stage in the development of bioengineered 3D tissue models.

Currently, *in vitro* fertilization (IVF) techniques are generally accepted, and as a result, surplus embryos destined to be discarded are mass-produced;

thus, relatively less ethical antipathy is associated with the limited research using these embryos. In some religious circles, the IVF technique that produces surplus embryos is regarded as ethically wrong. However, several people agree that surplus embryos, which cannot actually be discarded, are used for research to promote human well-being and health.^{39–44} However, the creation of embryos for application purposes, such as research, has received strong criticism because this strategy makes humans instrumental for specific purposes. In most cases, the purpose of donating sperm or eggs is to help infertile couples who cannot have babies in other ways.^{45,46} The consent of the donor is also limited such that if these donated materials are arbitrarily used to create an embryo, an ethical problem arises because the donor's intent is ignored. The donation of sperm or eggs for the purpose of research to create embryos is not accepted because germ cells, such as sperm and egg, are part of the body of the donor itself, and when combined and modified, these materials become completely different entities. The donation of reproductive cells with the knowledge that embryos may be created on an arbitrary basis using the donated sex cells or the use of these materials for such purposes is not ethically justifiable under the present social atmosphere. The whole living body and specific tissues, particularly germ cells, of humans should be treated carefully and thoughtfully.

Therefore, the study of human embryos has caused a great deal of ethical controversy, and some groups claim that the necessity of this research must be urgent for it to be justified. This idea is considered a balancing of benefits and harms and seeks answers for the following questions: “Are research studies on diseases that are expected to benefit greatly from embryo cloning, such as Parkinson's disease, diabetes, and myasthenia gravis, or cell development and stem cells that are important to our society worth the potential great harm to the well-being of its members?”, “Are other alternatives available to overcome these diseases?” and “Is there a scientific basis showing that this study will really help overcome these diseases?”.

If the answer to all these questions is yes, then these studies are justifiable, even at considerable ethical risks. Regarding the first question, a completely objective and rational answer cannot be obtained by reaching a consensus from members of society, as each society may reach different conclusions according to its historical experiences and cultural traditions. For example, if we conclude that building a nursing home to care for elderly people with Parkinson's disease and caring for these individuals as a social responsibility is better than sacrificing innocent human embryos, we should support these institutions and create regulatory measures. The answer to the second question is difficult to determine until a paradoxical study is performed showing that human embryos, umbilical cord stem cells, or adult stem cells, which are commonly referred to as alternatives to embryos, are more economical and effective. Therefore, further studies are necessary to examine potential alternatives. With respect to the third question, scientific research with a certain focus has historically resulted in many by-products and coincidental discoveries, rather than direct results. Therefore, these

unintended discoveries have often produced a more positive effect. Thus, when intensive research is conducted with a therapeutic purpose, the product in the process rather than the therapeutic drug or treatment method may produce greater contributions to science. By studying the differentiation of stem cells from the embryo, we can acquire extensive knowledge about cell differentiation, intercellular interactions, signal regulation, *etc.*, rather than obtaining stem cells that are immediately used for treatment, and this information may help researchers understand carcinogenesis and cellular immunity. Obviously, despite the positive and optimistic outlook, the negative view of the ethical issues concerning the use of human embryos for treatment is inevitable and cannot be resolved in a short time by unilateral persuasion. If the research on human embryos cannot be avoided in the process of researching and using stem cells, then this process must be achieved through a completely transparent and rigorous process, and the embryo should be treated with care and not as a simple thing but as a precious being with dignity.

16.2.1.2 Umbilical and Adult Stem Cells

Umbilical cord stem cells are generally included in the category of adult stem cells, but some ethical differences may occur. The ethical problem might not be as controversial because neither of the two methods requires cloning, human embryos, or the use of somatic cell nuclear transfer technology. In particular, adult stem cells are considered a type of autologous transplant if they are extracted from the body of the patient and cultured or differentiated; thus, the use of these cells is accepted as a usual treatment if management only at the intermediate stage is appropriate.

Adult stem cells, such as cells derived from blood and bone marrow, can have management problems, such as the possibility of infection. Notably, researchers are not able to guarantee that similar problems will not arise, such as the circulation of blood infected with AIDS, and unlike blood, the cells can be multiplied and used in many individuals, which can cause major social problems. Therefore, special attention should be paid to these problems if adult stem cells are used as actual treatments.

Umbilical cord stem cells are less likely to have problems with ownership and the possibility of infection, as the cord blood has been abandoned in the process of birth. The mother and her family typically do not claim any rights to the placenta, umbilical cord, and umbilical cord blood, which are generally discarded as medical waste.

Recently, however, an active movement to store the umbilical cord blood for the future of the child who has been born has received increasing attention, and social awareness related to this issue is increasing. In other words, if the umbilical cord blood is preserved, then this blood can be used for other family members or the treatment of childhood cancer or blood diseases that may occur in the future, and many hospitals and private companies are establishing cord blood banks.^{47–49} Therefore, in the future,

cord blood may not be considered a form of waste that the mother no longer needs and it will not be as easy to use at will. Thus, because cord blood is a resource that may be needed by the mother, the baby, and their families, the consent of the parties involved must be obtained to take advantage of this tissue.⁵⁰ Perhaps the most realistic approach is to receive informed consent from the mother after providing a full explanation of the use of umbilical cord blood. Umbilical cord blood is typically used for the following purposes: (1) unconditionally discarded, (2) stored in a cord blood bank for the family, (3) donated to a cord blood bank, and (4) donated for research purposes. If the mother or family does not understand each purpose, then the associated costs, the advantages and disadvantages, the existence of facilities at the delivery center, and the methods used to receive assistance should be extensively explained and consent should be obtained. If the cord blood is used for research purposes, detailed information describing who will use the blood and for what purpose must be provided, rather than obtaining general consent, in accordance with the consent form used in typical clinical studies.

16.3 Ethics Related to the Donation of Cells

A second ethical issue related to the use of human cells or tissues concerns the donation and collection of those cells or tissues. Several important issues associated with the donation of cells/tissues must be considered. As the number of the scientific studies using human samples has rapidly increased in recent years, concern that the privacy of the donors might be invaded has increased. The preservation of the donor's identity is the most important concern. The confidentiality of the donor data is considered a basic requirement for all activities using human samples. This confidentiality is typically achieved through the anonymization of the samples used in the scientific research.^{30,47,51–54} According to authors, free and unpaid donations are the ideal samples, based on the policy regulating the collection of samples for research, as payment may unduly induce vulnerable and poor living donors, constitute a conflict of interest for the next of kin or legal representatives in the donation of samples from deceased individuals, and likely results in inequities in donations.^{54–57}

Second, the donors should be clearly informed about the research in which the donated cells or tissues will be used in the future.^{30,33,51,58} Appropriate consent is based on the principle that competent individuals are entitled to freely choose whether to participate in research and should be given appropriate and sufficient information to be able to make this choice. The general consent form is considered acceptable if the following two conditions are fulfilled: the approval of all future projects by a research ethics committee, and the participants' right to withdraw samples at any time. Participants who are asked to consent to a donation should be properly informed, have the capacity to make the decision to participate without coercion or pressure, and understand their right to withdraw from

the research study at any time without providing a reason, and in the case of patients, without effects on their future medical care.^{30,51,52,58,59} Information should include the process involved in obtaining samples, any significant associated risks, and if known, the intended use of the samples and how the results of the research might impact their interests. Donors should be informed of intentions for the future storage and use of samples, including the potential sharing of samples with others. Human samples may vary according to the purpose for which they are collected. Different consent procedures are required to reflect the ethical differences between the removal of a sample as part of a person's therapy and the donation of a sample. The fate of donated samples may vary according to the intended use of the samples, as specified in the informed consent form.⁵⁴ The donor should be able to refuse consent. Donated cells or tissues should not be used without the consent of the donor. Thus, an obviously unethical situation will occur if the collected cells or tissues are used for purposes other than those described to the donor when consent was obtained. Good practice is to consider obtaining separate consent for the storage and use of human samples for research purposes wherever possible; when this type of consent is not reasonably possible, the appropriate information should be provided to ensure that patients are aware of the potential use of the samples.

Third, the question of the ownership of the body and its parts is complex in both ethical and legal terms. Ownership is occasionally interpreted as a question of who has authority over the use of the donated cells or tissues, and more frequently concerns whether the human body is subject to property rights.^{59–61} Currently, most global organizations and governments recognize that no person can own another person, as this ownership would constitute slavery and violate Article 4 of the Universal Declaration of Human Rights.⁶² The question of who has authority over the use of donated cells or tissues is more complicated.⁶³ The scenario is very different in the case of the body or parts of the body of a deceased individual that have been removed and treated or processed in some way. For example, people may feel a stronger association with a heart, brain or kidney made out of their own cells than simply using donated cells for that purpose.⁶⁴ Several legal cases have addressed the ownership of the body and its parts, and these cases help us to understand the current legal perspective regarding the legitimate removal of cells, tissues, and organs. Once the biological material has been removed from the donor, the recipient acquires the right to possession and use, regardless of whether the recipient is also the owner.⁶⁵ In the event that the recipient has also processed the material in some way, this individual can acquire an additional series of rights, including at least in some cases, a right of ownership. Ethically and legally, researchers cannot own a human body or its parts once they have been removed from the donor. Notably, although researchers cannot originally own the sample itself, they can 'own' the product of the work or skill applied to that sample.^{61,66,67}

16.4 Ethics Related to Clinical Trials

If cell therapy is applied to humans, then the criteria for a general clinical trial should be followed. Since scaffolds containing cells have not yet been established as a standard treatment, their safety has not been proven. Therefore, careful attention must be paid to all processes, including the selection of the subjects, obtaining consent from the subjects, the conduct of the research, the examination and announcement of the results, *etc.*^{30,62} A definitive report of a successful treatment using differentiated embryonic stem cells has not been published. Some experiments using fetal tissue showed only positive effects. In contrast, some animal experiments have reported the incidence of cancer (teratoma).^{30,68}

Additional studies are needed to determine whether the transplanted cells will exhibit the expected level of activity, whether they will differentiate into erroneous cells or cause malignant tumors, and how these cells will be affected if they are located within a scaffold during this process. Before this issue can be resolved to a certain extent, clinical trials on the human body should be avoided.^{69–72} Of course, trials in the human body will be justified if, at a minimum, safety is proven.

Preclinical studies should be conducted to verify safety. The aim of preclinical studies is to first determine the initial dose required in clinical trials and to provide the appropriate information needed to increase dosing.^{72–76} The target organ of the second cell therapy agent provides data to determine the toxicity and parameters for testing through clinical trials.

Finally, preclinical studies can determine the population of subjects at high risk for the toxicity of 3D-bioprinted tissue.^{22,64,77} In preclinical studies, (1) the population of cells to be administered; (2) the animal species and physiological conditions that best reflect the efficacy and the clinical efficacy of the product; and (3) the dosage plan, route of administration, and prescription must be reported. Appropriate animal models must be used to determine reproductive and developmental toxicity and carcinogenicity.^{20,78–80} Unlike other drugs, cell therapy works through interactions with living cells, and the results of animal models are not easy to extrapolate to humans.⁸¹ Therefore, even if a certain degree of safety is ensured in preclinical studies, these findings will be difficult to directly to humans due to differences among individuals and species. Therefore, assessments of the safety of cell therapy in preclinical studies must be more stringent and broader than for other drugs.

Another concern regarding the safety of 3D-bioprinted tissue, which differs from other drugs, is the need to thoroughly confirm the absence of infectious agents, such as viruses.²³ This step is a common procedure for biologically-produced drugs. Cells are susceptible to infections of microorganisms, such as viruses, during extraction, culture, and transfer; thus, care must be taken throughout the entire process. In particular, when microorganisms are derived from heterologous cells, microorganisms present in other species may cause unexpected infectious diseases.

After all these criteria are met, clinical trials can be conducted on patients. However, the use of this innovative treatment requires careful selection of subjects. In principle, subjects should be selected carefully by considering the benefits and risks of the treatment for the subject, a principle used for other standard and safe therapeutic options.⁸⁰ However, the appropriate results may be difficult to obtain in the clinical trial if these test treatments are confined to patients who are not available for all treatments. Therefore, researchers must fully comply with all principles of the clinical trial, after considering all relevant factors and ensuring patient voluntary participation. Subjects should be fully informed of the purpose, method, predictable outcomes and risks of the study, methods to address problems, and the responsible persons involved; the research ethics review committee should oversee this entire process.^{82–86} Only a sufficiently qualified researcher is able to conduct this type of research, and the patients' medical records and related information should only be available to researchers and clinicians who require access. Occasionally, a subject may need to be isolated for a period of time to prevent the spread of unknown infectious diseases, such as in the transplantation of xenogeneic cells, and measures should be taken to ensure the privacy and freedom of the individual. If any problem arises during the study, then the investigator or the research ethics review committee should immediately terminate the trial. However, unlike conventional drugs, 3D-bioprinted tissues require special precautions in preparation for this situation⁶⁵ since once the tissue is inserted into the body, it cannot be recycled unless the cells die by themselves.

16.4.1 Information and Consent

In bioprinting studies using stem cells, consent should be obtained from the embryo donor if the stem cells are derived from surplus embryos destined to be discarded.^{41,87} If adult and umbilical cord stem cells are used, consent should be obtained from the donor.^{50,88–90} Consent is required even when the study involves all human tissues, not just stem cells. The same principle applies to various human tissues, blood and by-products used in the research process. First, as the rights of the patient are expanded and the perception of patients' rights spreads, the organ or product derived from the body should not be used arbitrarily by others, regardless of the physician. Second, the development of life sciences has expanded the range of applications of human tissue products, and the economic value has greatly increased. Therefore, rights and obligations have been established, and the consent of the parties has become essential.⁹¹ Third, the development of genetic engineering and information technology has made it easier to grasp, search for, store and disseminate personal genetic information, which could seriously infringe on privacy, and obtain consent must be obtained prior to initiating the research. The classic elements of informed consent are divided into the following five categories.

16.4.1.1 Disclosure of Information

For information-based consent, the information must first be fully disclosed.⁹² Researchers must honestly disclose all details concerning the purpose of the research, the benefits obtained from the research, and the identities of the researchers, responsible agencies, and grant providers. In some cases, some of this information may not be able to be revealed to ensure the smooth conduct of the research, and in these cases, this information should be reviewed and approved by the regulatory body or the deliberation committee.

16.4.1.2 Comprehension of Information

The disclosure and provision of information does not mean that all parties involved understand it. Comprehension involves communication between the provider and recipient and is influenced by the intellectual abilities of both parties, the degree of education and background, and the cultural background and is also influenced by various peripheral factors at the time of communication.⁹³ In the case of a researcher who needs to obtain consent based on sufficient information, he/she has an obligation to continuously assess whether the subject has fully understood his/her explanations. Even under ideal circumstances, 100% content delivery is difficult to achieve, particularly when an individual must agree with a specific issue. The provider of the information should use easy expressions and generic terms, and the subject should be allowed sufficient time and space to repeat the question until all doubt is erased. In addition, the subject should clearly understand that disadvantages will not occur, even if he/she does not agree, and efforts should be made to reach a mutual consensus on subtle aspects, such as privacy violations and economic rights.

16.4.1.3 Voluntariness

For informed consent to be valid, consent must be obtained entirely on a voluntary basis.⁹⁴ Occasionally, spontaneity is difficult to maintain in the research environment when the subject is a student, assistant, *etc.* Concerns about academic and monetary loss may exist if the subject is a researcher, refuses the request and feels that he or she will not receive optimal treatment, or when the subject is a member of the research team and academic research is not conducted smoothly. Spontaneity may even be undermined in group inspections, such as studies of detainees, soldiers, and students who cannot sufficiently state their opinions. Therefore, when examining the subjects in this situation, researchers must pay particular attention to whether spontaneity is met. In addition, spontaneity is undermined when money or entertainment is provided in exchange for participation in the study, and monetary compensation should not be provided, except for the minimum amount of money necessary to conduct the research.

16.4.1.4 Competence

When the subject has a condition that renders them incompetent to make an informed decision, the validity of the consent based on sufficient information is questionable. Individuals with quasi-incompetency and incompetency due to intellectual disability or loss of mind and body, minors, and individuals with a low educational status may experience difficulty in understanding the context of the study.⁹⁵ Therefore, when obtaining consent, researchers should be aware of whether the subject meets the aforementioned criteria. In particular, for minors, researchers should determine whether these individuals represent the best interests of the study, even if consent can be obtained from legally valid representatives.

16.4.1.5 Consent

Valid consent is normally obtained in the form of a document. This document should contain the information and description of all relevant information, the address and name of the research institution and the name and address of the responsible persons.^{30,51,52,54,58,59} This agreement is only valid for the study described and must be re-certified by a new document when a new study is needed.

Occasionally, the blood or amniotic fluid obtained during a procedure are used anonymously for the purpose of the experiment. In other words, the sample is a type of by-product, and if it is not used for research, then it will be destroyed and not be related to the original owner in the experimental context.^{50,88–90} One example is when some blood plasma components are extracted from the donated blood and used for cell culture. In this case, a requirement to obtain consent from the original owner may be overly cumbersome, and thus the researchers are able to use the sample after review by the research ethics review committee. However, the process and procedure used to acquire the material must be readily identifiable, and researchers must confirm that the material has no relationship with the original owner in terms of human identity and privacy.

In addition, the validity period, procedures, and contents associated with consent can also be problematic in practice. In general, the consent is valid until the end of the study, and subjects are able to freely withdraw consent at any time in the middle of the study.⁹⁶ However, once the donation is made, due to the nature of the stem cell research, withdrawal is realistically difficult. In clinical trials, the person would not have to participate in the study. However, would the embryos or embryo-derived stem cells be able to be seized prior to the end of the study? Unlike other studies in which intangible knowledge obtained from research is the result of research, research on stem cells may result in the production of stem cell or differentiated cell lines. In addition, the validity of the withdrawal of consent will also not be easy to assert simply because consent was limited to the original embryo and not necessarily to any cells differentiated from the original cells using various

genetic manipulations and treatments. Considering these points, the embryo or tissue donor in the stem cell research may be in a disadvantageous position compared to the researcher, and further investigations are needed.

No perfect consent form can include all possible situations. Clearly, the consent form is not a universal document that allows the researcher all freedoms. However, the consent form is a minimum standard for securing the ethics of research and guarantees ethics on concrete matters based on the constant interaction with the research ethics review committee.

Finally, clinical trials using cell-based 3D bioprinting will likely require many years to track the results and side effects in contrast to trials testing other drugs. Thus, many years may be required to confirm that the therapeutic cells safely reach the target organs and determine whether transplantation produces the necessary effect and does not cause any other side effects. Therefore, the end point of the study needs to be adjusted accordingly, and researchers should be able to track the endpoint while the subject is alive. Researchers should be able to easily establish a research plan that carefully considers the effects of these conditions on the privacy of the participating individuals.

16.5 Conclusions

This article provided an exploration of the general ethical issues related to the use of 3D bioprinting; the ethical implications of 3D bioprinting are multifaceted and complex, particularly at the experimental trial stage. At this stage, the most important consideration is the protection of patients and society from the potential harm associated with this technology.

Optimistically, we may be able to avoid the moral implications related to the removal of organs and tissues from dead or live donors for transplantation, in addition to the previously mentioned creation of alternatives to animal testing. We could also avoid the problem of waiting lists that are too long compared to the needs of the patients, together with the difficulty in establishing appropriate criteria for the allocation of the few available organs. Furthermore, from a clinical perspective, the creation of organs with 3D bioprinting technology using cells obtained from a patient who needs a transplant could avoid the risks of rejection once the transplant has been performed.

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